Intraneural Application of Botulinum Neurotoxin A Improves Motoneuron Innervation and Functional Recovery after Femoral Nerve Reconstruction in Rats

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1. Abbreviations

$^{+}$	positive
°C	degrees Celsius
µg	microgram
µl	microliter
µm	micrometer
ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
BoNT	botulinum neurotoxin A
BSA	bovine serum albumin, bovines Serumalbumin
ChAT	choline acetyltransferase
CNS	central nervous system
Cy3	cyanine 3 dye
e.g	exempli gratia, for example
et al.	et alia, and others
FG	Fluoro-Gold
GABA	gamma-aminobutyric acid, Gamma-Aminobuttersäure
Hz	Hertz
i.e.	id est, that is
i.m.	intramuscular
IgG	immunoglobulin G
kg	kilogram
M	molar
mg	milligram
min	minute(s)
ml	milliliter
mm	millimeter
mM	millimolar
MW	molecular weight, molecular mass
N	number
N.	nervus
Nv	number per unit volume
PBS	phosphate-buffered saline
r^2	R squared, coefficient of determination
RT	room temperature
SFMA	single-frame motion analysis
SNAP-25	synaptosomal nerve-associated protein 25
trkB	tropomyosin receptor kinase B
U	unit(s)
VEGF	vascular endothelial growth factor
VGAT	vesicular GABA transporter
VGLUT1	vesicular glutamate transporter 1
VGLUT2	vesicular glutamate transporter 2

2. Summary

2.1 Background

Axonal injuries to motoneurons of adult mammals cause, among other responses, loss of synaptic terminals from their cell bodies and dendrites. This “synaptic stripping” is largely, but not always completely reversed after successful axonal regeneration and muscle reinnervation. Long-lasting deficits in, e.g., cholinergic and glutamatergic afferent terminals, correlate negatively with degree of functional recovery in rats suggesting that persistent partial deafferentation of motoneurons may be a factor limiting functional recovery after peripheral nerve injury.

2.2 Aims

To further explore the idea that functional recovery is partially linked to restoration of synaptic inputs to regenerated motoneurons, we pursued to modulate the deafferentation of motoneurons following nerve section/suture and monitor the effects of this manipulation on the outcome of peripheral nerve regeneration. Two neuroactive molecules, botulinum neurotoxin A (BoNT) and brain-derived neurotrophic factor (BDNF), known for their ability to influence synaptic inputs to neurons, were selected as manipulation tools.

2.3 Material and methods

Drug solutions were applied to the proximal stump of the freshly cut femoral nerve of rats to achieve drug uptake and transport to the neuronal perikarya and possibly transcytosis to afferent synapses. The experiments were performed in adult (10-week-old) female Wistar rats which received either BoNT (N = 17), BDNF (N = 15) or bovine serum albumin treatment (BSA, control, N = 13). After drug application, the femoral nerve was surgically reconstructed and functional recovery was monitored over a 5-month period using an established gait analysis protocol. Other outcome measures were quality of endplate reinnervation (occurrence of abnormal polyinnervation assessed morphologically) and numbers of cholinergic, GABA/glycinergic and glutamatergic synaptic terminals in the femoral motor nucleus in the spinal cord (assessed using stereological approaches).

2.4 Results and discussion

Application of BoNT, but not BDNF, resulted in a marked, as compared with BSA, improvement of motor recovery at 2 to 20 weeks after injury. At two months, BoNT-treated rats had an attenuated loss of perisomatic cholinergic terminals compared with the other two treatments. Analysis of individual animal data revealed significant linear correlations between functional parameters and numbers of cholinergic terminals. Quality of endplate reinnervation was not affected by treatment with BoNT or BDNF. The effect of BoNT on synaptic terminals is possibly related to transcytosis of BoNT into perisomatic nerve terminals as suggested by immunohistochemical analysis of cleaved SNAP-25. In turn, better preservation of modulatory cholinergic terminals, which are crucial for normal motoneuron excitability, might underlie enhanced recovery of function in BoNT-treated rats.

2.5 Conclusions

The findings support the idea that persistent partial deafferentation of axotomized motoneurons is a factor contributing to deficient functional recovery after nerve injury. Intraneural application of drugs appears to be a promising way to analyze causal relationships between synaptic plasticity and restoration of function. In addition, it is thinkable that the described drug application approach may evolve into a clinically feasible therapy if further controlled animal experiments provide convincing evidence for its safety and efficacy.

2. Zusammenfassung

2.1 Hintergrund

Läsionen peripherer Nerven von erwachsenen Säugetieren verursachen Verlust von Synapsen an Zellkörpern und Dendriten von Motoneuronen. Diese Deafferenzierung ("synaptic stripping") ist, selbst nach erfolgreicher Nervenregeneration und Reinnervation der Muskulatur, nicht immer vollkommen reversibel. Langfristige Defizite sind für cholinerge und glutamaterge Afferenzen nachgewiesen und diese korrelieren negativ mit dem Grad der funktionellen Erholung bei Ratten. Es kann angenommen werden, dass eine persistierende partielle Deafferenzierung von Motoneuronen das funktionelle Outcome einer peripheren Nervenverletzung beeinträchtigen kann.

2.2 Fragestellungen

Um die o. g. Hypothese zu überprüfen, wurde hier versucht die Deafferenzierung der Motoneurone nach Durchtrennung und Naht des Nervus femoralis der Ratte zu manipulieren. Zwei neuroaktive Moleküle, Botulinum-Neurotoxin A (BoNT) und Brain-derived neurotrophic factor (BDNF), die bekanntlich synaptische Eingänge an Neuronen beeinflussen, wurden als „Manipulationswerkzeuge" ausgewählt. Die Effekte dieser Behandlungen auf das Outcome der Nervenregeneration wurden funktionell und strukturell untersucht.

2.3 Material und Methoden

BoNT oder BDNF wurden an den proximalen Stumpf des durchtrennten Nervus femoralis appliziert, um ihre Aufnahme und den Transport zu den Perikarya sowie eventuell die Transzytose zu afferenten Synapsen zu erreichen. Als Kontrolle diente bovines Serumalbumin (BSA). Die Experimente wurden an erwachsenen, zehn Wochen alten weiblichen Wistar Ratten durchgeführt, die entweder eine BoNT (N = 17), BDNF (N = 15) oder BSA (N = 13) Behandlung erhielten. Nach der Applikation wurde der Nerv chirurgisch rekonstruiert und die funktionelle Erholung mithilfe eines etablierten Ganganalyseverfahrens in einem Zeitraum von fünf Monaten dokumentiert. Weitere Outcome-Parameter waren die Qualität der Endplattenreinnervation (Auftreten anormaler Polyinnervation) und die Anzahl der

cholinergen, GABA/glycinergen und glutamatergen synaptischen Terminale im motorischen Kern des N. femoralis im Rückenmark.

2.4 Ergebnisse und Diskussion

Zwei bis 20 Wochen nach der Läsion war die funktionelle Erholung nach BoNT Applikation signifikant besser im Vergleich zu den anderen beiden Behandlungen. Der posttraumatische Verlust an perisomatischen cholinergen Synapsen zwei Monate nach der Operation war ebenso reduziert in BoNT-behandelten Tieren. Regressionsanalysen zeigten signifikante lineare Korrelationen zwischen Funktionsparametern und der Anzahl cholinerger Terminale. Die Qualität der Endplattenreinnervation wurde nicht durch die Behandlung mit BoNT oder BDNF beeinflusst. Die Effekte von BoNT beruhen, wie durch Nachweis von gespaltetem SNAP-25 in perisomatischen Nerventerminale angedeutet, möglicherweise auf BoNT Transzytose. Eine bessere Erhaltung der modulatorischen cholinergen Terminale, die für die normale Erregbarkeit von Motoneuronen von entscheidender Bedeutung sind, könnte wiederum einer verbesserten Erholung der Funktion bei mit BoNT behandelten Ratten zugrunde liegen.

2.5 Schlussfolgerungen

Die Ergebnisse stützen die Hypothese, dass langanhaltende partielle Deafferenzierungen von Motoneuronen negative Auswirkungen auf die funktionelle Erholung nach Nervenläsionen haben können. Die intraneurale Anwendung von neuroaktiven Substanzen scheint ein vielversprechender Weg zu sein, um kausale Zusammenhänge zwischen synaptischer Plastizität und Wiederherstellung der Funktion zu analysieren. Es ist außerdem denkbar, dass sich der beschriebene Ansatz der Arzneimittelapplikation zu einer klinisch durchführbaren Therapie entwickeln kann, wenn weitere kontrollierte Tierversuche überzeugende Beweise für seine Sicherheit und Wirksamkeit liefern.

3. Introduction

3.1 Synaptic stripping

Peripheral axotomy in adult laboratory animals and humans causes loss of synaptic terminals from cell bodies and dendrites, a phenomenon known as synaptic stripping (Blinzinger and Kreutzberg, 1968; Graeber et al., 1993). The synaptic terminal displacement is mediated by activated microglia and supported by astrocytes that isolate the postsynaptic elements by thin lamellae (Blinzinger and Kreutzberg, 1968; Kerns and Hinsman, 1973a,b; Sumner and Sutherland, 1973; Chen, 1978; Kreutzberg, 1993; Borke et al., 1995). In general, it is considered that the posttraumatic loss of synapses is a temporary phenomenon which is completely reversed as reinnervation proceeds and muscles become reinnervated (Sumner and Sutherland, 1973; Sumner, 1975; Chen, 1978). However, a few publications have reported that restoration of particular synaptic inputs is incomplete, namely of inhibitory F-type (containing flat vesicles) terminals on reinnervated cat medial gastrocnemius motoneurons (Brännström and Kellerth, 1999) and proprioceptive Ia afferent synapses (VGLUT1-immunoreactive boutons) on rat tibialis motoneurons (Rotterman et al., 2014; Schultz et al., 2017).

Physiologically, the acute post-traumatic deafferentation is believed to be a response protecting the axotomized, and thus transmission disabled motoneurons against excitotoxicity (Spejo and Oliveira, 2015). The functional consequences of reafferentation deficits after muscle reinnervation have received little attention. One exception is the hypothesis that permanent loss of Ia afferent synapses and failure to restore the stretch reflex essentially contribute to poor functional recovery after spinal nerve injury and repair (Rotterman et al., 2014; Schultz et al., 2017).

3.2 Synapses as variables relevant for functional recovery

Recent studies in our laboratory have suggested that functional limitations after successful muscle reinnervation may in part be attributed to deficient reafferentation of motoneurons (Hundeshagen et al., 2013; Raslan et al., 2014). Using the facial nerve model and nerve lesions of different severity (“mild” cryolesion and “severe” section/suture), the studies revealed that degree of functional recovery, estimated by quantitative whisker motion analysis, is positively correlated, on an individual basis, with numbers of persisting cholinergic and glutamatergic terminals in the motor

nucleus following long-term reinnervation. The suggested structure-function relationships are plausible since glutamatergic and cholinergic synapses determine drive and excitability of motoneurons, respectively (Miles et al., 2007; Heckman et al., 2009; Raslan et al., 2014).

3.3 Modulation of motoneuron innervation

The next logical step on the way to prove a causal relationship between synaptic numbers and functional outcome was to manipulate the independent variable, synapses, and monitor changes in behavior. In this study we pursued to influence synaptic responses after peripheral nerve injury by using BoNT and BDNF. The reasons for this choice were as follows:

3.3.1 BoNT

Upon intramuscular application, BoNT blocks synaptic transmission at the neuromuscular junction and, in addition, is transported retrogradely to the motoneuron cell body and possibly also transcytosed to afferent synaptic terminals (Antonucci et al., 2008; Matak et al., 2012; Restani et al., 2012a,b). BoNT causes progressive synaptic stripping detectable at 4 days after intramuscular injection and abolishes excitatory and inhibitory synaptic transmission on motoneurons at 1-2 weeks after application (Pastor et al., 1997). Rather than intramuscularly, we applied the drug to the proximal nerve stump immediately after nerve transection similar to the application of retrograde tracers. The rationale behind this unusual type of application was three-fold: 1/ to avoid possible involvement of the periphery (muscle), which could blur the results, 2/ to eventually achieve high (though not measurable!) drug concentrations in the cell body and 3/ create a model relevant for potential clinical application (possibility to apply drug only to the severed nerve prior to surgical reconstruction in humans). The expectation was that BoNT will significantly enhance synaptic stripping.

3.3.2 BDNF

With an intraneural application similar to BoNT, we expected to achieve opposite effects using BDNF, i.e. attenuation of synaptic loss. When administered to the proximal axons immediately after transection, BNDF reduces synaptic stripping and enhances recovery of tonic firing of regenerating motoneurons (Davis-Lopez de

Carrizosa et al., 2009). Synaptotrophic effects of exogenous BDNF have also been reported after ventral root avulsion (Novikov et al., 2000). Finally, a single session of brief electrical stimulation (20 Hz, 1 hour) of the proximal stump of the freshly transected femoral nerve in rats leads to enhanced nerve regeneration over weeks and this effect is apparently associated with an up-regulation of BDNF and its cognitive receptor trkB in the motoneuron cell body (Al-Majed et al., 2000a,b). It is plausible that enhanced BDNF signaling is beneficial for regeneration via synaptotrophic effects.

3.4 The experimental model

The femoral nerve model in rats was selected for this first experiment using intraneural drug application. The anatomy in this model allows work with a longer proximal trunk after nerve transection as compared with, for example, the facial nerve and, thus, easier and more secure drug application using plastic mini-cups. The well-established femoral nerve model in rodents is a valuable alternative to other spinal nerve models like the sciatic one offering the possibility to analyze precision of target reinnervation, reliable functional assessments and a straightforward search of anatomical deficits and structure-function correlations (Irintchev, 2011). A very helpful background was also the existence of extensive data on long-term functional recovery, precision of motor reinnervation and correlations between these measures after suture/section of the femoral nerve in adult rats (Kruspe et al., 2014). The outcome measures used in this study included: 1/ motor recovery test (single-frame motion analysis, SFMA), 2/ stereological estimates of chemically defined nerve terminal densities in the femoral motor nucleus and 3/ retrograde labeling of motoneurons. Whenever possible and meaningful, both functional and structural analyses were performed on one and the same animals to provide basis for correlations.

4. Aims

This work was designed to test the hypothesis that exogenous drugs can modulate injury-induced synaptic rearrangements in the femoral motor nucleus of adult rats and, as a consequence, alter the functional outcome.

A first step towards a successful experimental solution was to test if synaptic stripping can be modulated using the selected model, type of drug application and drug concentrations. The results of this experiment (Experiment I in 5.), in which I was not directly involved, showed that one week after injury and drug application BoNT leads to a reduced loss of GABA/glycinergic terminals and both BoNT and BDNF increase the numbers of remaining cholinergic perisomatic synaptic terminals (data in Fig. 3 in 5.). These results clearly showed the feasibility of the project and warranted further progression of the work focused on the following specific aims:

1. To test the long-term (at 2 months post-lesion) functional effects after femoral nerve transection, application of BoNT, BDNF or BSA (control) to the proximal nerve trunk and subsequent nerve reconstruction (Experiment II in 5.). The method of choice here was single-frame motion analysis (SFMA), an established approach for functional analyses after spinal nerve lesions.

2. To test, in the same animal groups, the long-term effects of the drugs on numbers of inhibitory (GABA/glycinergic), excitatory (glutamatergic) and modulatory (cholinergic) terminals and microglia in the femoral motor nucleus of the spinal cord. The method of choice here was stereological assessment of synaptic terminal and cell densities using the optical disector method, a routinely used approach in our laboratory.

3. To analyze the long-term functional effects (over 5 months) of BoNT, BDNF and BSA application using the SFMA approach (Experiment III in 5.).

4. To analyze the precision of motor reinnervation in the animal groups mentioned above (point 3.) using retrograde labeling of correctly and incorrectly regenerated motoneurons, i.e., via the femoral and saphenous nerve branch, respectively. The back-labeling method is a routine technique in the laboratory.

5. To correlate functional and structural data using regression analysis.

5. Publication

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Research Article

Botulinum Neurotoxin Application to the Severed Femoral Nerve Modulates Spinal Synaptic Responses to Axotomy and Enhances Motor Recovery in Rats

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Botulinum neurotoxin A (BoNT) and brain-derived neurotrophic factor (BDNF) are known for their ability to influence synaptic inputs to neurons. Here, we tested if these drugs can modulate the deafferentation of motoneurons following nerve section/suture and, as a consequence, modify the outcome of peripheral nerve regeneration. We applied drug solutions to the proximal stump of the freshly cut femoral nerve of adult rats to achieve drug uptake and transport to the neuronal perikarya. The most marked effect of this application was a significant reduction of the axotomy-induced loss of perisomatic cholinergic terminals by BoNT at one week and two months post injury. The attenuation of the synaptic deficit was associated with enhanced motor recovery of the rats 2–20 weeks after injury. Although BDNF also reduced cholinergic terminal loss at 1 week, it had no effect on this parameter at two months and no effect on functional recovery. These findings strengthen the idea that persistent partial deafferentation of axotomized motoneurons may have a significant negative impact on functional outcome after nerve injury. Intraneural application of drugs may be a promising way to modify deafferentation and, thus, elucidate relationships between synaptic plasticity and restoration of function.

1. Introduction

Injury to peripheral nerves in adult mammals causes deafferentation of the axotomized motoneurons, a phenomenon known as "synaptic stripping" [1]. Synaptic terminals are removed from cell bodies and dendrites of motoneurons by activated microglial and astroglial cells [1–6]. The overall posttraumatic loss is reversed to a large extent if muscles become reinnervated [3, 6, 7], but restoration of some synaptic inputs is incomplete [8–11]. Such deficits, for example, in cholinergic and glutamatergic innervation, may contribute to functional deficits after muscle reinnervation as they are well correlated with functional performance after long-term reinnervation [9, 12].

Here, we pursued to influence synaptic responses after peripheral nerve injury and, thus, eventually alter the outcome by using botulinum neurotoxin A (BoNT) or brain-derived neurotrophic factor (BDNF). When applied intramuscularly, BoNT blocks synaptic transmission at the neuromuscular junction and, in addition, is transported retrogradely to the motoneuron cell body and possibly also transcytosed to afferent synaptic terminals [13–16]. BoNT causes progressive synaptic stripping detectable at 4 days after intramuscular injection and abolishes excitatory and inhibitory synaptic transmission on motoneurons at 1-2 weeks after application [17]. Rather than intramuscularly, we applied BoNT to the proximal nerve stump immediately after nerve transection similar to the application of retrograde tracers assuming that this type of application will enhance synaptic stripping similar to intramuscular BoNT application. In other animals, we applied BDNF to the proximal stump of the freshly cut nerve hoping to achieve an effect opposite to that of BoNT, that is, attenuation of synaptic loss. When administered to cut proximal axons immediately after transection, BNDF reduces synaptic stripping and enhances recovery of tonic firing of regenerating motoneurons [18].

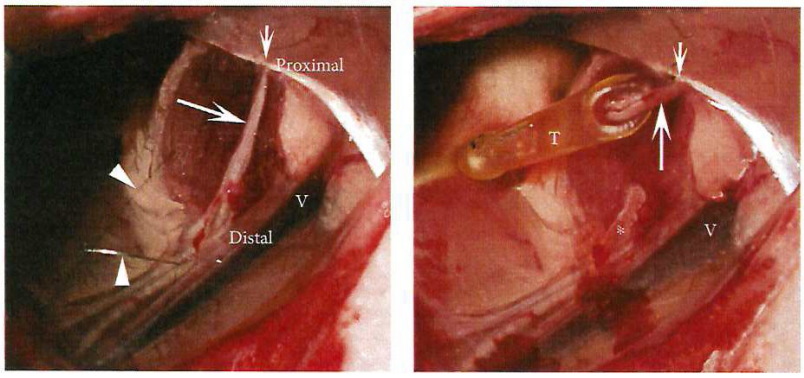
Synaptotrophic effects of exogenous BDNF have also been reported after ventral root avulsion [19]. Finally, a single session of brief electrical stimulation (20 Hz, 1 hour) of the proximal stump of the freshly transected femoral nerve in rats leads to enhanced nerve regeneration over weeks and this effect is apparently associated with an upregulation of BDNF and its cognitive receptor TrkB in the motoneuron cell body [20, 21]. It is possible, though not proven, that this enhanced BDNF signaling leads to, among other mechanisms, better regeneration via synaptotrophic effects. We measured the effects of BoNT or BDNF application using stereological estimates of chemically defined nerve terminal densities in motor nuclei, a motor recovery test, and retrograde labeling of motoneurons. For this first experiment using intraneural drug application, we selected the femoral nerve model in rats for a practical reason: the anatomy in this model allows work with a longer proximal trunk after nerve transection as compared with, for example, the facial nerve and, thus, easier application of BoNT or BDNF solutions to the severed nerve using plastic mini cups. The well-established femoral nerve model is a valuable alternative to other spinal nerve models like the sciatic one offering the possibility to analyze precision of target reinnervation, reliable functional assessments, and a straightforward search of anatomical deficits and structure-function correlations [22]. Helpful for this study was also previous data on long-term functional recovery, precision of motor reinnervation, and correlations between these measures after section/suture of the femoral nerve in adult rats [23].

2. Materials and Methods

2.1. Animals and Experimental Design. Ten-week-old female Wistar Unilever rats ($N = 65$) from Charles River Laboratories (Sulzfeld, Germany) were used. To monitor short-term numerical changes in synaptic terminal populations, retrograde neuronal tracer (Fluoro-Gold, FG) was injected unilaterally into the quadriceps muscles of 20 animals (experiment I). Four days later, the femoral nerve on the injected side was cut and solutions containing bovine serum albumin (BSA), BoNT, or BDNF were applied to the proximal nerve stump (5 rats per group, see details on application below). Synaptic populations in the quadriceps motor nucleus, defined by the retrograde labeling, were studied one week after nerve transection. The rest five rats served as an "intact" control, that is, they were similarly treated and analyzed with the exception of nerve injury. To analyze long-term synaptic alterations, the rats in experiment II were subjected to nerve lesion and application of BSA ($N = 6$), BoNT type A ($N = 7$), or BDNF ($N = 7$). Intramuscular (i.m.) injections of FG were performed two months after injury followed by, one week later, video recordings for single-frame motion analysis (SFMA) and tissue sampling for synaptic terminal analyses. Analysis of long-term functional effects was done in experiment III. After nerve injury and application of BSA ($N = 7$), BoNT ($N = 10$), or BDNF ($N = 8$), the animals were repeatedly video recorded over a 20-week observation period and then subjected to retrograde labeling of motoneurons regenerated beyond the injury site to analyze "preferential motor reinnervation" [24]. The animals were housed under standard conditions and received food and water ad libitum. Visual examinations for complications like BoNT-induced muscle paralysis, abnormal grooming, or self-mutilations were performed regularly (once daily in the first week, once or twice weekly at later time periods). Such complications were not observed. Experiments were performed according to the animal protection laws of Germany and the European Community. Experiments were blinded.

2.2. Surgery and Drug Application. Rats were anesthetized with fentanyl (Fentanyl Janssen, Janssen, Neuss, Germany, 0.005 mg/kg i.m.), midazolam (Dormicum-R, Roche, Basel, Switzerland, 2 mg/kg i.m.), and medetomidine (Domitor-R, Orion Pharma, Espoo, Finland, 0.15 mg/kg i.m.). The trunk of the right nerve was exposed under an operation microscope and cut at approximately 7 mm proximal to the bifurcation of the saphenous and quadriceps muscle branches (Figure 1(a)). The proximal nerve stump was inserted for 30 min into a cup containing 0.1% BSA (Sigma, Taufkirchen, Germany) in saline, 100 U/ml BoNT (Xeomin, Merz Pharma, Frankfurt, Germany), or 20 μg/ml human recombinant BDNF (Biomol, Hamburg, Germany) in 0.1% BSA saline (Figure 1(b)). As a rough orientation for the drug concentrations served previous in vivo studies on synaptic effects using BoNT [13, 17] and BDNF [18]. The cups were cut from standard yellow pipette tips after their distal ends were heat-sealed using a lighter (Figure 1(b), capacity ~10 μl). After drug treatment, the nerve trunks and their surroundings were thoroughly rinsed with saline and the nerve ends were aligned using two epineural 10–0 sutures (Ethicon, Norderstedt, Germany). Finally, the skin was closed with 4–0 sutures (Ethicon) and the rats received subcutaneously an antidote cocktail consisting of atipamezole (Antisedan, Orion Pharma, 0.75 mg/kg), flumazenil (Anexate, Roche, 0.2 mg/kg), and naloxone (Naloxon, CuraMed Pharma, Karlsruhe, Germany, 0.12 mg/kg).

2.3. Single-Frame Motion Analysis (SFMA). SFMA was performed as described previously [23]. Briefly, the rats (experiments II and III) were video recorded prior to nerve injury from behind and from the left and right side during walking along a wooden plate (1500 mm long, 120 mm wide, and 20 mm thick) using a video camera (100 frames per second, Pike F-032, Allied Vision Technologies, Stadtroda, Germany). The video recordings were repeated 8 weeks (experiment II) or at 1, 2, 4, 8, 12, 16, and 20 weeks (experiment III) after injury. At least three walking trials were recorded per rear, left and right side view of each animal per time point. Analyses were performed using noncommercial software packages: VirtualDub 1.6.19 (http://www.virtualdub.org) and Image Tool 3.0 (University of Texas Health Science Center at San Antonio, TX, USA, http://compdent.uthscsa.edu/imagetool.asp). Two parameters were measured: the foot-base angle (FBA) and the step length ratio (SLR). The FBA is measured at toe-off position on the side ipsilateral to injury as an angle between the line dividing the sole surface



(a) (b)

FIGURE 1: Drug application to the severed nerve. (a) The right femoral nerve trunk (arrow) prior to nerve injury. Proximally, the nerve is fixed by an epineural suture (short arrow) to the nearby muscle aponeurosis to prevent withdrawal of the proximal stump after nerve cut. Seen are also the 10–0 thread (upper arrowhead) used to fix the nerve and its needle (lower arrowhead), as well as the femoral vein (V). (b) The femoral nerve is transected, and the proximal stump is inserted in a self-made cup (T, see Materials and Methods) filled with drug solution. The distal nerve stump is marked by an asterisk.

into two halves and the horizontal line (minimum of 3 measurements per animal and time point). The SLR is calculated as ratio of the lengths of two successive steps (minimum of 6 SLR values per animal and time point). Using the FBA and SLR values, two additional parameters were calculated: (1) the product FBA × SLR and (2) the FBA × SLR recovery index [23].

2.4. Retrograde Labeling of Motoneurons. To label the quadriceps motor nucleus (experiments I and II), 125 μl of 1% Fluoro-Gold (Fluorochrome, Denver, CO, USA) in saline was injected into the right quadriceps muscle without anesthesia of the rats (Figure 2(a)). For analysis of "preferential motor reinnervation" [23], 20 weeks after injury, the rats in experiment III were anesthetized as described above. The quadriceps and the saphenous branches were cut approximately 5 mm distal to the bifurcation. Fluoro-Ruby (tetramethylrhodamine dextran, MW 10,500, Molecular Probes/Life Technologies, Darmstadt, Germany) and Fluoro-Emerald (fluorescein dextran, MW 10,000, Molecular Probes) crystals were applied for 30 min to the proximal stumps of the quadriceps and the saphenous branch, respectively. Labeling was considered successful if no leakage of dye beyond the parafilm sheaths underlying the nerve ends was noticed after the 30 min application period. Six days later, the rats were anaesthetized and perfused with 4% formaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3. The lumbar spinal cords were removed, postfixed overnight, and cut transversely (serial sections of 40 μm thickness) on a cryostat (CM1850, Leica Microsystems, Wetzlar, Germany). The sections were collected on SuperFrost Plus glass slides (Carl Roth, Karlsruhe, Germany) and coverslipped using Fluoromount G (Southern Biotechnology Associates/Biozol, Eching, Germany). Counting was based on stereological principles and done on an Axiophot 2 fluorescence microscope [25].

2.5. Immunofluorescence. Tissue processing and staining were performed as previously described [26]. Under anesthesia (see above), the rats were perfused with 4% formaldehyde in 0.1 M cacodylate buffer, pH 7.3, for 15 min at room temperature (RT). The lumbar spinal cords were then postfixed in the same fixative overnight at 4°C and cryoprotected by infiltration with 15% sucrose in cacodylate buffer for 2 days at 4°C. The samples were frozen in precooled 2-methylbutane (isopentane, −80°C) for 2 min and stored in liquid nitrogen until sectioned. Transverse sections of 25 μm thickness were obtained using a cryostat (CM1850, Leica Microsystems, Wetzlar, Germany) such that 6 spaced serial sections 250 μm apart were present on each slide. Immunofluorescence staining was performed after antigen retrieval (30 min at 80°C in 10 mM sodium citrate solution, pH 9.0). Nonspecific binding was blocked for 1 hour at RT with phosphate-buffered saline (PBS, pH 7.3) containing 0.2% Triton X-100 (Sigma), 0.02% sodium azide (Sigma), and 5% normal serum (Jackson ImmunoResearch Europe, Suffolk, UK) from the species in which the secondary antibody was raised (Table 1). The primary antibodies were diluted in PBS containing 0.5% lambda-carrageenan (Sigma) and 0.2% sodium azide and applied to the sections for 3 days at 4°C (Table 1). Cy3-conjugated secondary antibodies, diluted in PBS containing 0.5% lambda-carrageenan and 0.2% sodium azide, were applied for 2 hours at RT (Table 1). Cell nuclei were stained for 10 min at RT with bis-benzimide solution (Hoechst 33258 dye, 5 μg ml^{-1} in PBS, Sigma). For each antigen, all sections were stained in the same primary and secondary antibody solutions stabilized by the nongelling vegetable gelatin lambda-carrageenan and kept in screw-capped staining plastic jars (capacity 35 ml, 10 slides, Carl Roth). This method enables repeated long-term usage and high reproducibility of the immunohistochemical staining [26–28]. Staining controls included omitting the first antibody or replacing it by normal serum or IgG. These controls

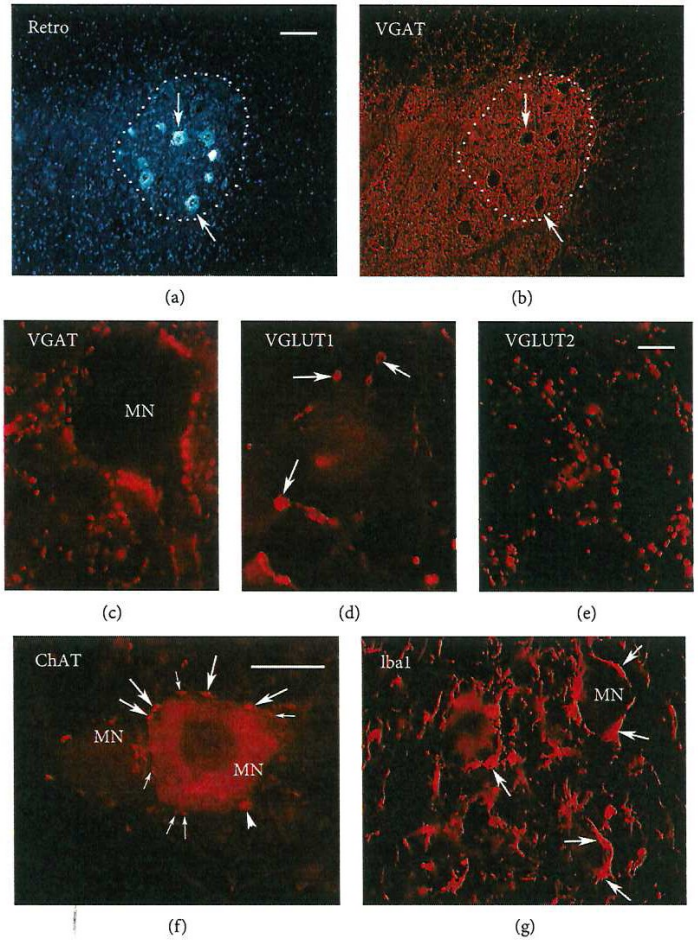


FIGURE 2: Images of synaptic terminals and Iba1$^+$ cells in the quadriceps motor nucleus. (a-b) A section containing back-labeled cell bodies of femoral motoneurons (a, arrows) is additionally stained for nuclei (a) and VGAT (b). The boundary of the quadriceps motor nucleus is indicated by a dotted line. Scale bar = 100 μm for (a-b). (c–e) VGAT$^+$ and VGLUT 2$^+$ axonal terminals (c, e) and VGLUT1$^+$ varicosities (arrows, d). Scale bar = 10 μm for (c–e). (f) ChAT staining of two motoneuron cell bodies (MN) surrounded by cholinergic terminals (arrows). Counted were terminals around the MN soma with a visible nucleus (pale area in the center of the MN on the right hand side) which were in focus (thick arrows). Terminals out of focus or only partially seen in the focus plane (thin arrows) were not counted. No quantification was undertaken for the second MN profile (on the left hand side) since it had no visible nucleus. The arrowhead points to a ChAT$^+$ cross-sectional profile of a dendrite close to the MN cell body. Such "perisomatic" dendritic profiles could be traced for long distances throughout the section thickness in contrast to the limited extent of the perisomatic terminals in the z-axis. (g) Iba1$^+$ cells (arrows) some of which surround a motoneuron cell body (MN). Scale bar indicates 25 μm and 50 μm for panels (f) and (g), respectively. (a–g) Shown are representative images from tissue sections after different treatments to illustrate the quality of each staining which was similar in all experimental groups and time points.

were negative. Examples of immunohistochemical stainings are shown in Figures 2(b)–2(g).

2.6. Quantitative Immunohistochemical Analyses. Quantitative analyses were performed using the Stereo Investigator 8.1 software (MicroBrightField Europe, Magdeburg, Germany) and a fluorescence microscope (Axioskop 2 mot plus, Zeiss, Oberkochen, Germany) equipped with a motorized stage (Zeiss) and a CX 9000 digital camera (MicroBrightField) as described [9, 12]. Cell and synaptic terminal densities were estimated using the optical disector in every 10th spaced serial section (250 μm apart) in which back-labeled femoral motoneurons were visible (Figure 2(a)). The boundaries of the quadriceps motor nucleus were outlined (Plan Neofluar 5x objective, Zeiss, Figure 2(a)), and cell or synaptic terminal densities (N_v) were estimated using randomly placed disectors. For VGAT$^+$ (Figures 2(b) and 2(c)), VGLUT1$^+$ (Figure 2(d)), and VGLUT2$^+$ terminals (Figure 2(e)), the disectors had a 100 μm^2 base and a 5 μm height with an interdisector spacing of 100 μm. Individually

Table 1: Antibodies used for immunohistochemistry.

Antigen	Species and type, dilution	Supplier, code	Structures labeled by primary antibodies	References
Choline acetyltransferase	Goat polyclonal, 1 : 500	Chemicon/Millipore, Schwalbach, Germany, AB144P	Cholinergic cells, axons and axon terminals, large perisomatic terminals on motoneurons	Hellström et al. [44], Nagy et al. [45], Wilson et al. [46]
Iba1 (ionized calcium binding adaptor molecule 1)	Rabbit polyclonal, 1 : 1500	Wako Chemicals, Neuss, Germany, 019-19741	Microglial cells	Imai et al. [64], Ito et al. [65]
VGAT (vesicular GABA transporter)	Mouse monoclonal, 1 : 500	Synaptic Systems, Gottingen, Germany, 131 011	Inhibitory (GABAergic and glycinergic) axon terminals	Chaudhry et al. [66], McIntire et al. [67], Wojcik et al. [68]
VGLUT1 (vesicular glutamate transporter 1)	Rabbit polyclonal, 1 : 1000	Synaptic Systems, 135 303	Excitatory (glutamatergic) axon terminals of primary (Ia) afferents	Alvarez et al. [69], Oliveira et al. [70], Rotterman et al. [10]
VGLUT2 (vesicular glutamate transporter 2)	Rabbit polyclonal, 1 : 1000	Synaptic Systems, 135 403	Excitatory (glutamatergic) axon terminals of spinal cord interneurons	Alvarez et al. [69], Oliveira et al. [70]
SNAP-25 BoTox-A cleaved	Mouse monoclonal (4F3-2C1), 1:200	MyBioSource, San Diego, CA, USA, MBS350064	Synaptic terminals containing SNAP-25 (synaptosomal-associated protein 25) cleaved by botulinum toxin A	Manufacturer's data sheet, Rheaume et al. [32]
Goat IgG	Cy3-conjugated donkey polyclonal, 1 : 200	Jackson ImmunoResearch Europe, Suffolk, UK, 705-165-003		
Mouse IgG	Cy3-conjugated goat polyclonal, 1 : 200	Jackson ImmunoResearch, 115-165-003		
Rabbit IgG	Cy3-conjugated goat polyclonal, 1 : 200	Jackson ImmunoResearch, 111-165-003		

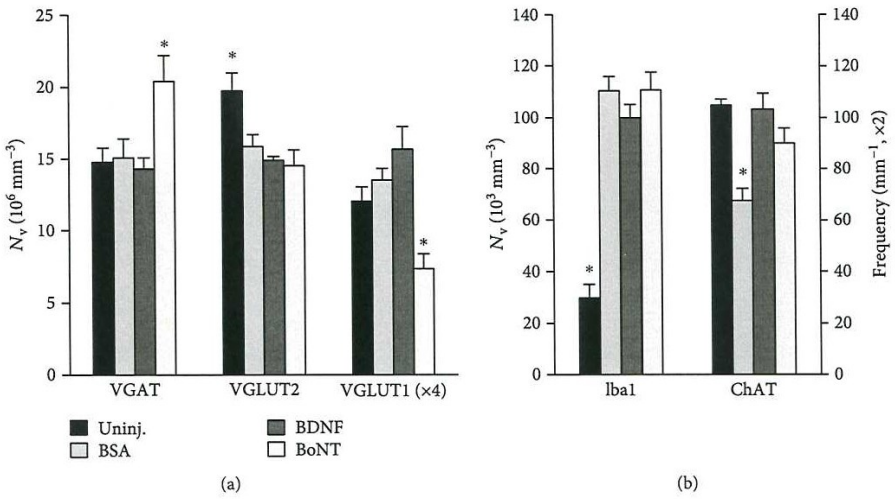


FIGURE 3: Analysis of synaptic terminals and microglia in the quadriceps motor nucleus 1 week after femoral nerve injury and drug application. Included are also values from control rats without nerve injury and drug treatment ("Uninj."). Shown are numerical densities (number per unit volume) of VGAT$^+$, VGLUT1$^+$, and VGLUT2$^+$ terminals and Iba1$^+$ microglial cells, as well as frequency (number per unit length) of ChAT$^+$ perisomatic terminals (mean values + SEM). Asterisks indicate mean values significantly different from all other groups (one-way ANOVA, $F_{3,16} = 4.87–44.8$, $p = 0.014 - <0.001$) with Holm-Sidak post hoc tests ($p = 0.042 - <0.001$). $N = 5$ per group.

discernible immunopositive puncta were counted using a Plan Neofluar 100x oil objective (Zeiss). For Iba1$^+$ cells (Figure 2(g)), the size of the disectors was 3600 μm^2 base and 10 μm height and the spacing between disectors was 100 μm.

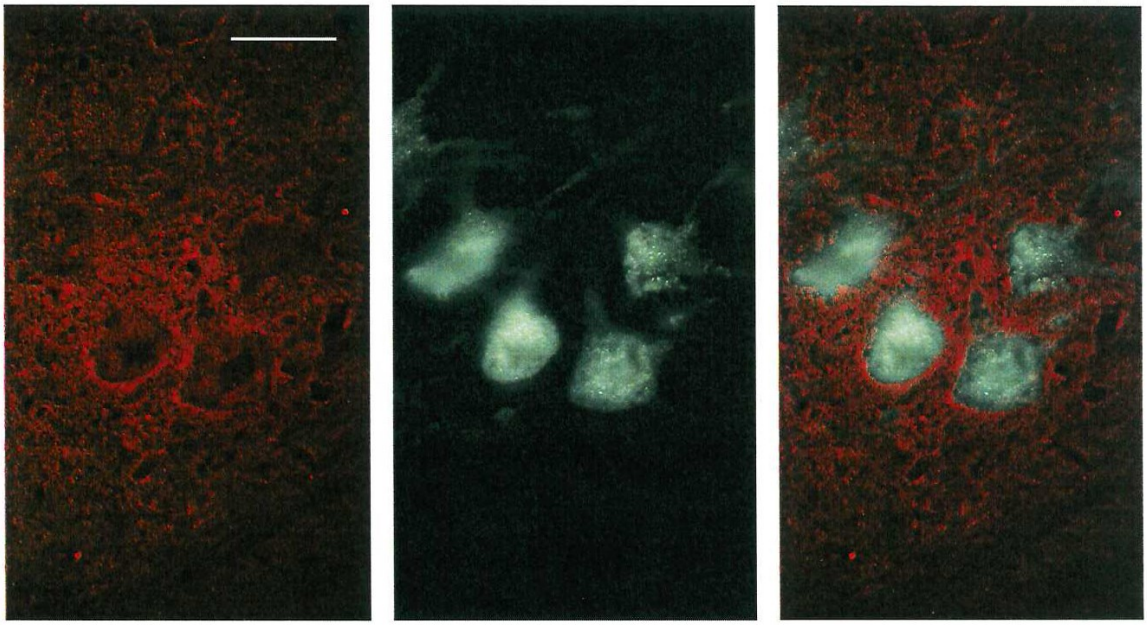
Analyses of cholinergic perisomatic terminals were performed on ChAT-immunostained sections using the Stereo Investigator (Figure 2(f), [9]). All motoneuron profiles with discernible nucleus in a quadriceps motor column transect were analyzed. Each motoneuron, visualized at 100x magnification, was focused at the level of its largest cell body cross-sectional area, and its cell body perimeter and number of perisomatic terminals were determined (Figure 2(f)). Frequency of perisomatic ChAT$^+$ terminals was calculated as number of perisomatic terminals per unit perimeter length. Mean values of individual animals were used to calculate group mean values.

2.7. Statistical Analyses. Data were analyzed using one-way analysis of variance (ANOVA) or two-way ANOVA for repeated measures followed by Holm-Sidak multiple comparison tests (SigmaPlot 12, SPSS, Chicago, IL, USA). Regression analyses were performed using SigmaPlot. The threshold value for acceptance of differences was 5%.

3. Results and Discussion

3.1. Short-Term Effects on Synaptic Terminal Numbers. We initially tested whether intraneural drug applications alter short-term synaptic responses to nerve injury in the spinal motor nucleus (experiment I). We estimated the effects of nerve injury and application of BSA as compared to rats without nerve lesions ("BSA" versus "Uninj." in Figure 3) using antibodies against synaptic terminal markers (Table 1). Numbers of microglial cells were also analyzed since these cells are activated after injury and are involved in synaptic remodeling [29–31]. The observed effects included reduced density of excitatory VGLUT2$^+$ terminals (−20%, Figure 3(a)), increased density of Iba1$^+$ microglia (+267%, Figure 3(b)), and decrease in modulatory perisomatic ChAT$^+$ terminals (−36%, Figure 3(b)). Inhibitory VGAT$^+$ and excitatory VGLUT1$^+$ Ia boutons were not significantly affected (+2% and +13%, resp., Figures 3(a) and 3(b)). Assuming that BSA has no measurable influence on these variables, the differences found between the two groups represent axotomy-related responses. In line with this notion is the finding of similar changes in the rat facial nucleus 1 week after axotomy [9]. Compared with BSA, BDNF had only one effect: attenuation of injury-induced ChAT$^+$ terminal loss (Figure 3(b)). A similar protective effect on ChAT$^+$ terminals had also BoNT (Figure 3(b)). In addition, BoNT application resulted, again as compared with BSA, in increased density of VGAT$^+$ terminals (+35%) and reduced density of VGLUT1$^+$ boutons (−46%), while VGLUT2$^+$ terminals and Iba1$^+$ cells were not significantly affected (−9% and 0%, resp., Figure 3(a)).

To test if the BoNT effects could be related to its retrograde transport into the spinal cord, we performed immunohistochemistry for BoNT-cleaved SNAP-25 (SNAP-25$_{197}$) which labels sites of BoNT proteolytic activity [32]. One week after nerve injury and BoNT application, immunofluorescence labeling was present around back-labeled somata and in the neuropil of the femoral motor nucleus (Figure 4). This pattern of labeling is similar to that previously observed by



(a) (b) (c)

FIGURE 4: Cleaved SNAP-25 staining of a spinal cord section one week after injury and BoNT application. Immunostaining (a) is seen around the somata of back-labeled motoneurons and in the neuropil among them (b, c). Scale bar = 50 μm.

other groups [13, 14] and suggests that BoNT action has been transported into the spinal cord and could possibly be active in afferent terminals.

Overall, these findings show that the drug applications altered some synaptic responses to axotomy. Our working hypothesis was (see Introduction) that BDNF would have synaptotrophic effects and, indeed, injury-related loss of $ChAT^+$ perisomatic boutons was prevented. At the same time, however, other major inputs, excitatory $VGLUT2^+$ and inhibitory $VGAT^+$ terminals, were not affected as initially hypothesized. It is possible that the intracellular concentration of active exogenous BDNF achieved in our experiment has not been optimal to produce pronounced, long-term effects. BDNF appears to have a dose-dependent influence on nerve regeneration, that is, facilitation at low doses and inhibition at higher ones [33]. Therefore, we do not assume that BDNF is inefficient in our model unless this proves true in a future dose-dependence study.

In contrast to BDNF, we expected that BoNT would enhance loss of terminals after axotomy with a more pronounced effect on excitatory ($VGLUT1^+$ and $VGLUT2^+$) than on inhibitory ($VGAT^+$) terminals [34, 35]. This appeared true for $VGLUT1^+$ terminals, but the effects on $VGAT^+$ and $ChAT^+$ terminals were, on the opposite, synaptotrophic (Figure 3). This heterogeneity of effects suggests also other mechanisms of action in addition to inhibition of synaptic vesicle exocytosis by cleaving SNAP-25 [35]. It is possible, for example, that the increase in inhibitory $VGAT^+$ terminals results from inhibition of some of these heterogeneous in origin terminals [36] and subsequent sprouting of unaffected inhibitory axons. Partial inhibition and reactive sprouting could also affect the cholinergic input to motoneurons. Alternatively or in addition, it is possible that BoNT has neurotrophic effects achieved via colocalization and signaling through the p75 receptor [15, 37]. This notion is not necessarily in disagreement with the limited effects of BDNF described above since different receptors (p75 versus TrkB) and neurotrophins may be involved.

3.2. Long-Term Synaptic Effects and Recovery of Function. We further investigated whether drug-related synaptic alterations persist after a longer reinnervation period, two months after injury (experiment II). We found, again compared with a BSA control group, that the BDNF effect on $ChAT^+$ terminals at 1-week post injury has disappeared while a previously nonexisting deficit in $VGLUT1^+$ terminals was now present (Figures 5(a) and 5(b)). BoNT-related differences in $VGAT^+$ and $VGLUT1^+$ terminal numbers had also disappeared at two months after injury, but the $ChAT^+$ terminal frequency was still higher similar to 1 week after lesion (Figures 5(a) and 5(b)). Immunohistochemistry for cleaved SNAP-25 in the spinal cord at two months after injury showed labeling similar to the one observed at 1 week (data not shown). This observation suggests that BoNT enzymatic activity is present for a long period of time after application.

Functional analysis performed in the same animal groups revealed significantly lower foot-base angle (FBA) and step length ratio (SLR) in the BoNT group as compared to BSA- and BDNF-treated rats (Figure 6(a)). This finding indicates better functional recovery as both parameters increase after

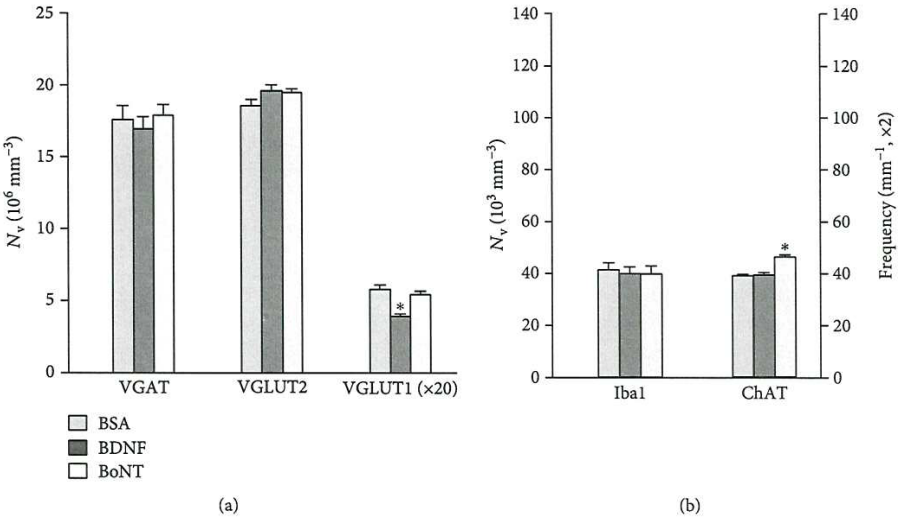


FIGURE 5: Analysis of synaptic terminals and microglia in the quadriceps motor nucleus two months after femoral nerve injury and drug application. Asterisks indicate mean values significantly different from all other groups (one-way ANOVA, $F_{2,16} = 11.4$ and 30.4, $p < 0.002$ and 0.001 for VGLUT1 and ChAT, resp.) with Holm-Sidak post hoc tests ($p = 0.005 - <0.001$). $N = 5 - 7$ per group. Note that numbers of Iba1$^+$ cells and ChAT$^+$ terminals (b) and numbers of VGLUT1$^+$ terminals (a) in BSA-treated animals are much lower than these at 1 week after injury (Figures 3(a) and 3(b)). This is consistent with previous findings [9, 10].

injury and decrease as reinnervation and recovery proceed (see Figures 7(a) and 7(b)). Regression analysis did not indicate any significant statistical relationship between individual structural parameters (Figure 5) and functional measures (Figure 6(a)) with the exception of ChAT$^+$ terminal densities (Figures 6(b)–6(d)). Higher frequencies of cholinergic perisomatic terminals appeared to be associated with lower ("better") functional values. The coefficients of determination (r^2, values shown in Figures 6(b)–6(d)) indicate that some 70% of the variability in functional parameters may be explained, in statistical terms, by variability in numbers of ChAT$^+$ terminals. Previous work using facial nerve or spinal cord injury models has also shown strong statistical relationships between degree of functional recovery, on one side, and degree of preservation/recovery of ChAT$^+$ terminal frequency on facial [9, 12] or spinal motoneurons [38–40], on the other side. These large cholinergic terminals form C-type synapses on motoneuronal perikarya and proximal dendrites and utilize M2 muscarinic receptors for acetylcholine in the postsynaptic membrane [41–46]. Although not that numerous, these synapses strongly influence motoneuron function by regulating action potential after hyperpolarization in a way that, under normal conditions, ensures sufficient motoneuron output to drive motor behavior [47, 48]. We can, therefore, assume that partial loss of perisomatic cholinergic terminals, associated with a reduced expression of postsynaptic receptors [49, 50], may significantly impair motor behaviors such as walking, whisking, and blinking [51].

3.3. Long-Term Functional Effects. Finally, we were interested whether functional effects of drug application could appear later or earlier than the analyzed postinjury time point (two months), a time period when reinnervation and recovery are well advanced but not completed. We performed experiment III in which rats were treated similarly to experiment II but monitored functionally between the first and the 20th week after injury. Time course and degree of recovery were very similar between BSA- and BDNF-treated animals (Figures 7(a)–7(d)) and in agreement with previous observations after transection and suture of the femoral nerve in adult rats [23]. In contrast, recovery after BoNT application was accelerated between the 2nd and 12th week (Figures 7(a)–7(d)) and advantages of this treatment were even present at the final time point studied, 20 weeks (Figure 7(a)).

After the 20-week observation period, the animals in experiment III were subjected to retrograde labeling to assess precision of reinnervation (Figures 8(a)–8(c)), a factor that can influence the functional outcome after femoral nerve injury and regeneration in rats [23]. The numbers of motoneurons projecting into the appropriate quadriceps nerve only, into the inappropriate saphenous nerve, or into both nerves ("Muscle," "Skin," and "Both" in Figure 8(d), resp.) were similar in the three groups of rats. This finding suggests that the functional improvements seen in the BoNT group are not related to an enhanced preferential reinnervation of the muscle. This notion is supported by the lack of significant covariations between numbers of back-labeled motoneurons and functional parameters.

3.4. Possible Mechanisms of Drug Effects. We applied BoNT only once using the time frame between axonal membrane damage and sealing to load the proximal axon and cell body

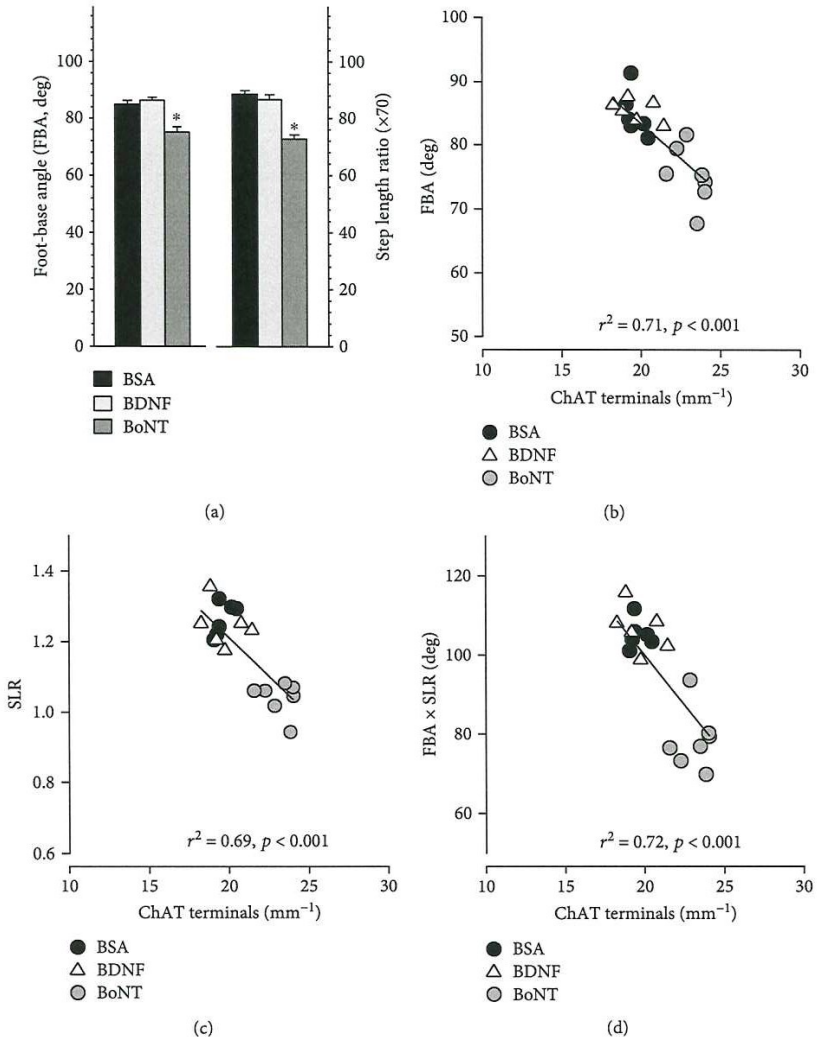


FIGURE 6: Motor recovery and correlations between functional parameters and ChAT terminal frequency two months after femoral nerve lesion and drug application. (a) Shown are mean values + SEM of foot-base angle (FBA) on the operated side and step length ratio (SLR). $N = 6$, 7, and 7 for BSA, BDNF, and BoNT, respectively. For both parameters, one-way ANOVA showed effects of treatment ($F_{2,17} = 18.4$ and 38.0 for FBA and SLR, respectively, $p < 0.001$ for both parameters). The BoNT group mean values were significantly different from the values of the BSA and BDNF groups (asterisks, $p < 0.001$, Holm-Sidak test). (b–d) Individual values of functional parameters plotted against numbers of ChAT terminals. Shown are regression lines, coefficients of determination (r^2), and probability values (p).

with toxin similar to retrograde tracers (Figures 8(a)–8(c)). Our expectation was that this uptake will be sufficient to "prime" the initial responses of motoneurons to injury, in particular their deafferentation, and, thus, eventually achieve long-term effects on regeneration without need of repeated drug delivery to the injury site. As estimated by gait analysis, our experiment was successful as functional regeneration was enhanced already at two weeks after injury and recovery remained accelerated for months thereafter. Enhancement of axonal regrowth in the crushed sciatic nerve of mice by a single low-dose intraneural application of BoNT has been just reported, but the underlying mechanisms for these effects have remained unclear [37]. Here, we propose that the improvement of regeneration in our model is a consequence of attenuated loss of cholinergic modulatory input to femoral motoneurons (Table 2). In addition, it is possible that BoNT has an additional neuroprotective effect. At one week after injury, we found, compared with control rats, an increase in VGAT$^+$ inhibitory afferents in the quadriceps motor nucleus, reduced numbers of excitatory VGLUT1$^+$ Ia afferents, and no change in excitatory VGLUT2$^+$ terminals (Figure 3, Table 2). We can speculate that this constellation

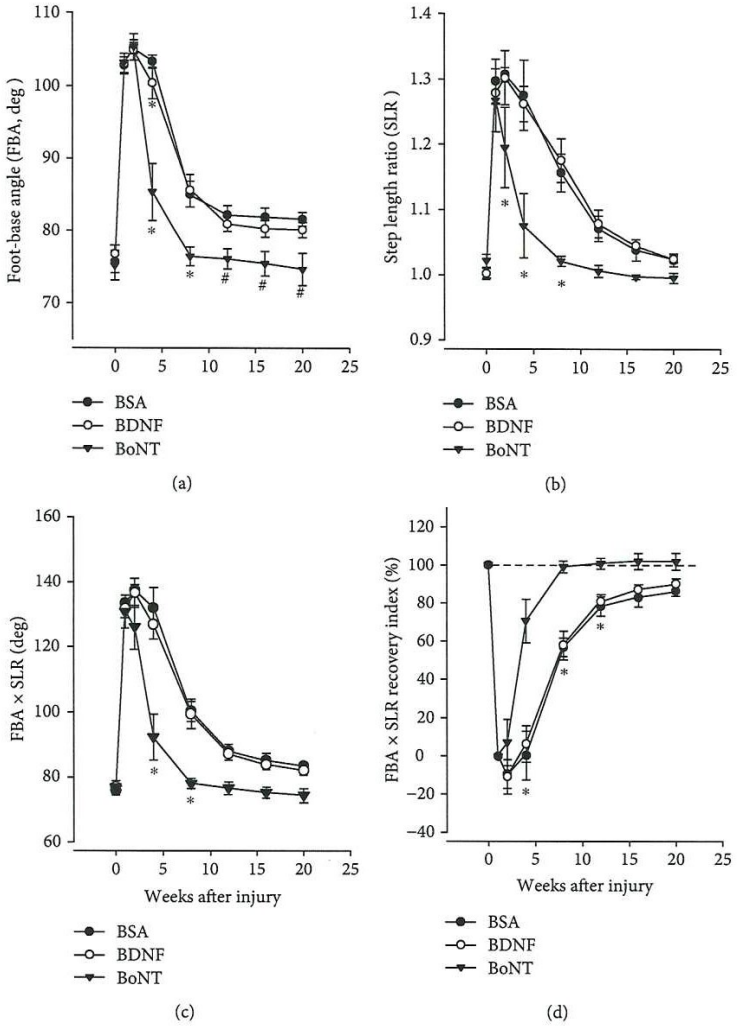


FIGURE 7: Time course and degree of motor recovery after femoral nerve lesion and drug application. Shown are mean values ± SEM of foot-base angle on the operated side (FBA, a), step length ratio (SLR, b), product FBA × SLR (c), and recovery index for the product FBA × SLR (d) prior to injury (0 week) and 1–20 weeks p.o. The dashed horizontal line in (d) is drawn at 100%, a value indicating full degree of recovery. $N = 7$, 8, and 9 for BSA, BDNF, and BoNT, respectively. For all parameters shown, two-way ANOVA for repeated measures showed effects of time ($F_{7,147} = 52.4–209$, $p < 0.001$) and treatment ($F_{2,21} = 9.51–15.6$, $p = 0.003 - <0.001$). Indicated by symbols are group mean values significantly different from * the corresponding postoperative values of the BSA and BDNF groups and # the corresponding value of the BSA group ($p < 0.05$, Holm-Sidak post hoc procedure).

attenuates the increased excitability of the axotomized motoneurons and, thus, allows better recovery of the motoneuron and its better regeneration [18, 52]. It is also thinkable that BoNT-related modulations of reflexes and/or pain-related transmission may have also positive functional consequences [53–56]. A major unresolved issue in this study is why BoNT had synaptotrophic effects on some types of synapses. The unexpected observation, which is unrelated to the main goal and achievement of this work, has to be explained by future experiments.

Similar to BoNT, BDNF is retrogradely transported from the periphery to the cell body of motoneurons and then transcytosed to afferent presynaptic terminals [57]. Exogenous BDNF has already shown synaptotrophic properties in injury models [18, 19, 58], and exogenous BDNF can improve axonal regeneration [59, 60]. We indeed found a BDNF effect at one week after injury—prevention of injury-induced ChAT$^+$ terminal loss (Figure 3, Table 2), but no functional effects were seen (Figures 6(a) and 7). This may be related to lack of a prolonged protective effect on ChAT$^+$

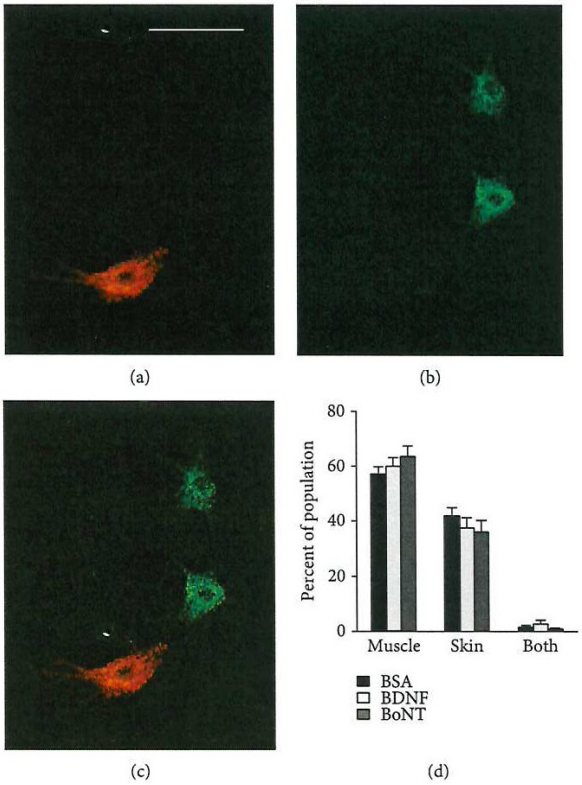


FIGURE 8: Retrograde labeling of motoneurons 20 weeks after lesion. (a–c) Representative images of motoneurons back-labeled through the muscle (quadriceps) and the skin (saphenous) branch of the femoral nerve ("Muscle" and "Skin") using Fluoro-Ruby and Fluoro-Emerald (red and green fluorescence), (a) and (b), respectively, overlay in (c). Scale bar = 100 μm. (d) Quantitative analysis of retrogradely labeled cells including double-labeled motoneurons ("Both"). Shown are mean values + SEM. One-way ANOVA showed no effect of treatment on any of the motoneuron categories ($F_{2,18} = 0.95–1.14$, $p = 0.342–0.533$). $N = 7$ animals per group.

TABLE 2: Summary of effects of drug application on VGLUT1$^+$, VGLUT2$^+$, ChAT$^+$, and VGAT$^+$ synaptic terminals and Iba1$^+$ cells one week and two months after injury. Arrows indicate increase (↑), decrease (↓), or no difference (=) compared to BSA treatment.

	BoNT versus BSA		BDNF versus BSA	
	1 week	2 months	1 week	2 months
VGLUT1	↓	=	=	↓
VGLUT2	=	=	=	=
ChAT	↑	↑	↑	=
VGAT	↑	=	=	=
Iba1	=	=	=	=

terminals as observed two months after BoNT application (Figure 5, Table 2).

4. Conclusions

The results of this study provide further support to the notion that insufficient recovery of synaptic inputs to motoneurons, in particular, perisomatic cholinergic terminals, may be an essential factor limiting recovery after peripheral nerve injury and regeneration. In addition, it appears encouraging that single intraoperative application of drugs to the severed nerve can be a useful way to modify neuronal responses to axotomy and, thus, modulate regeneration and eventually improve functional outcome of nerve injury. The list of candidates for such applications may be long, ranging from other neurotrophins or combinations of neurotrophins (e.g., BDNF and neurotrophin-3 [18], NGF [61]) or growth factors (e.g., vascular endothelial growth factor (VEGF) [62]) to small bioactive molecules [63].

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Acknowledgments

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References

[1] K. Blinzinger and G. Kreutzberg, "Displacement of synaptic terminals from regenerating motoneurons by microglial cells," *Zeitschrift für Zellforschung und Mikroskopische Anatomie*, vol. 85, no. 2, pp. 145–157, 1968.

[2] R. C. Borke, R. S. Bridwell, and M. E. Nau, "The progression of deafferentation as a retrograde reaction to hypoglossal nerve injury," *Journal of Neurocytology*, vol. 24, no. 10, pp. 763–774, 1995.

[3] D. H. Chen, "Qualitative and quantitative study of synaptic displacement in chromatolyzed spinal motoneurons of the cat," *The Journal of Comparative Neurology*, vol. 177, no. 4, pp. 635–663, 1978.

[4] J. M. Kerns and E. J. Hinsman, "Neuroglial response to sciatic neurectomy. I. Light microscopy and autoradiography," *Journal of Comparative Neurology*, vol. 151, no. 3, pp. 237–253, 1973.

[5] G. W. Kreutzberg, "Dynamic changes in motoneurons during regeneration," *Restorative Neurology and Neuroscience*, vol. 5, no. 1, pp. 59-60, 1993.

[6] B. E. H. Sumner and F. I. Sutherland, "Quantitative electron microscopy on the injured hypoglossal nucleus in the rat," *Journal of Neurocytology*, vol. 2, no. 3, pp. 315–328, 1973.

[7] B. E. H. Sumner, "A quantitative analysis of boutons with different types of synapse in normal and injured hypoglossal nuclei," *Experimental Neurology*, vol. 49, no. 2, pp. 406–417, 1975.

[8] T. Brännström and J. O. Kellerth, "Recovery of synapses in axotomized adult cat spinal motoneurons after reinnervation into muscle," *Experimental Brain Research*, vol. 125, no. 1, pp. 19–27, 1999.

[9] A. Raslan, P. Ernst, M. Werle et al., "Reduced cholinergic and glutamatergic synaptic input to regenerated motoneurons after facial nerve repair in rats: potential implications for recovery of motor function," *Brain Structure & Function*, vol. 219, no. 3, pp. 891–909, 2014.

[10] T. M. Rotterman, P. Nardelli, T. C. Cope, and F. J. Alvarez, "Normal distribution of VGLUT1 synapses on spinal motoneuron dendrites and their reorganization after nerve injury," *The Journal of Neuroscience*, vol. 34, no. 10, pp. 3475–3492, 2014.

[11] A. J. Schultz, T. M. Rotterman, A. Dwarakanath, and F. J. Alvarez, "VGLUT1 synapses and P-boutons on regenerating motoneurons after nerve crush," *The Journal of Comparative Neurology*, vol. 525, no. 13, pp. 2876–2889, 2017.

[12] G. Hundeshagen, K. Szameit, H. Thieme et al., "Deficient functional recovery after facial nerve crush in rats is associated with restricted rearrangements of synaptic terminals in the facial nucleus," *Neuroscience*, vol. 248, pp. 307–318, 2013.

[13] F. Antonucci, C. Rossi, L. Gianfranceschi, O. Rossetto, and M. Caleo, "Long-distance retrograde effects of botulinum neurotoxin A," *The Journal of Neuroscience*, vol. 28, no. 14, pp. 3689–3696, 2008.

[14] I. Matak, P. Riederer, and Z. Lackovic, "Botulinum toxin's axonal transport from periphery to the spinal cord," *Neurochemistry International*, vol. 61, no. 2, pp. 236–239, 2012.

[15] L. Restani, F. Giribaldi, M. Manich et al., "Botulinum neurotoxins A and E undergo retrograde axonal transport in primary motor neurons," *PLoS Pathogens*, vol. 8, no. 12, article e1003087, 2012.

[16] L. Restani, E. Novelli, D. Bottari et al., "Botulinum neurotoxin A impairs neurotransmission following retrograde transynaptic transport," *Traffic*, vol. 13, no. 8, pp. 1083–1089, 2012.

[17] A. M. Pastor, B. Moreno-Lopez, R. R. De La Cruz, and J. M. Delgado-Garcia, "Effects of botulinum neurotoxin type A on abducens motoneurons in the cat: ultrastructural and synaptic alterations," *Neuroscience*, vol. 81, no. 2, pp. 457–478, 1997.

[18] M. A. Davis-López de Carrizosa, C. J. Morado-Diaz, J. J. Tena et al., "Complementary actions of BDNF and neurotrophin-3 on the firing patterns and synaptic composition of motoneurons," *The Journal of Neuroscience*, vol. 29, no. 2, pp. 575–587, 2009.

[19] L. N. Novikov, L. N. Novikova, P. Holmberg, and J.-O. Kellerth, "Exogenous brain-derived neurotrophic factor regulates the synaptic composition of axonally lesioned and normal adult rat motoneurons," *Neuroscience*, vol. 100, no. 1, pp. 171–181, 2000.

[20] A. Al-Majed, T. Brushart, and T. Gordon, "Electrical stimulation accelerates and increases expression of BDNF and trkB mRNA in regenerating rat femoral motoneurons," *The European Journal of Neuroscience*, vol. 12, no. 12, pp. 4381–4390, 2000.

[21] A. A. al-Majed, C. M. Neumann, T. M. Brushart, and T. Gordon, "Brief electrical stimulation promotes the speed and accuracy of motor axonal regeneration," *The Journal of Neuroscience*, vol. 20, no. 7, pp. 2602–2608, 2000.

[22] A. Irintchev, "Potentials and limitations of peripheral nerve injury models in rodents with particular reference to the femoral nerve," *Annals of Anatomy*, vol. 193, no. 4, pp. 276–285, 2011.

[23] M. Kruspe, H. Thieme, O. Guntinas-Lichius, and A. Irintchev, "Motoneuron regeneration accuracy and recovery of gait after femoral nerve injuries in rats," *Neuroscience*, vol. 280, pp. 73–87, 2014.

[24] T. Brushart, "Preferential reinnervation of motor nerves by regenerating motor axons," *The Journal of Neuroscience*, vol. 8, no. 3, pp. 1026–1031, 1988.

[25] O. Simova, A. Irintchev, A. Mehanna et al., "Carbohydrate mimics promote functional recovery after peripheral nerve repair," *Annals of Neurology*, vol. 60, no. 4, pp. 430–437, 2006.

[26] A. Irintchev, A. Rollenhagen, E. Troncoso, J. Z. Kiss, and M. Schachner, "Structural and functional aberrations in the cerebral cortex of tenascin-C deficient mice," *Cerebral Cortex*, vol. 15, no. 7, pp. 950–962, 2005.

[27] A. Irintchev, M. Zeschnigk, A. Starzinski-Powitz, and A. Wernig, "Expression pattern of M-cadherin in normal, denervated, and regenerating mouse muscles," *Developmental Dynamics*, vol. 199, no. 4, pp. 326–337, 1994.

[28] M. V. Sofroniew and U. Schrell, "Long-term storage and regular repeated use of diluted antisera in glass staining jars for increased sensitivity, reproducibility, and convenience of single- and two-color light microscopic immunocytochemistry," *The Journal of Histochemistry and Cytochemistry*, vol. 30, no. 6, pp. 504–511, 1982.

[29] S. Cullheim and S. Thams, "The microglial networks of the brain and their role in neuronal network plasticity after lesion," *Brain Research Reviews*, vol. 55, no. 1, pp. 89–96, 2007.

[30] D. Gomez-Nicola and V. H. Perry, "Microglial dynamics and role in the healthy and diseased brain: a paradigm of functional plasticity," *The Neuroscientist*, vol. 21, no. 2, pp. 169–184, 2015.

[31] G. W. Kreutzberg, "Microglia: a sensor for pathological events in the CNS," *Trends in Neurosciences*, vol. 19, no. 8, pp. 312–318, 1996.

[32] C. Rheaume, B. B. Cai, J. Wang et al., "A highly specific monoclonal antibody for botulinum neurotoxin type A-cleaved SNAP25," *Toxins*, vol. 7, no. 7, pp. 2354–2370, 2015.

[33] M. Richner, M. Ulrichsen, S. L. Elmegaard, R. Dieu, L. T. Pallesen, and C. B. Vaegter, "Peripheral nerve injury modulates neurotrophin signaling in the peripheral and central nervous system," *Molecular Neurobiology*, vol. 50, no. 3, pp. 945–970, 2014.

[34] C. Verderio, C. Grumelli, L. Raiteri et al., "Traffic of botulinum toxins A and E in excitatory and inhibitory neurons," *Traffic*, vol. 8, no. 2, pp. 142–153, 2007.

[35] C. Verderio, D. Pozzi, E. Pravettoni et al., "SNAP-25 modulation of calcium dynamics underlies differences in GABAergic and glutamatergic responsiveness to depolarization," *Neuron*, vol. 41, no. 4, pp. 599–610, 2004.

[36] P. H. Beske, S. M. Scheeler, M. Adler, and P. M. McNutt, "Accelerated intoxication of GABAergic synapses by botulinum neurotoxin A disinhibits stem cell-derived neuron networks prior to network silencing," *Frontiers in Cellular Neuroscience*, vol. 9, p. 159, 2015.

[37] S. Cobianchi, J. Jaramillo, S. Luvisetto, F. Pavone, and X. Navarro, "Botulinum neurotoxin A promotes functional recovery after peripheral nerve injury by increasing regeneration of myelinated fibers," *Neuroscience*, vol. 359, pp. 82–91, 2017.

[38] I. Apostolova, A. Irintchev, and M. Schachner, "Tenascin-R restricts posttraumatic remodeling of motoneuron innervation and functional recovery after spinal cord injury in adult mice," *The Journal of Neuroscience*, vol. 26, no. 30, pp. 7849–7859, 2006.

[39] I. Jakovcevski, J. F. Wu, N. Karl et al., "Glial scar expression of CHL1, the close homolog of the adhesion molecule L1, limits recovery after spinal cord injury," *The Journal of Neuroscience*, vol. 27, no. 27, pp. 7222–7233, 2007.

[40] H. J. Lee, I. Jakovcevski, N. Radonjic, L. Hoelters, M. Schachner, and A. Irintchev, "Better functional outcome of compression spinal cord injury in mice is associated with enhanced H-reflex responses," *Experimental Neurology*, vol. 216, no. 2, pp. 365–374, 2009.

[41] Z. Csaba, E. Krejci, and V. Bernard, "Postsynaptic muscarinic m2 receptors at cholinergic and glutamatergic synapses of mouse brainstem motoneurons," *The Journal of Comparative Neurology*, vol. 521, no. 9, pp. 2008–2024, 2013.

[42] M. S. Davidoff and A. P. Irintchev, "Acetylcholinesterase activity and type C synapses in the hypoglossal, facial and spinal-cord motor nuclei of rats. An electron-microscope study," *Histochemistry*, vol. 84, no. 4-6, pp. 515–524, 1986.

[43] J. Hellström, U. Arvidsson, R. Elde, S. Cullheim, and B. Meister, "Differential expression of nerve terminal protein isoforms in VAChT-containing varicosities of the spinal cord ventral horn," *The Journal of Comparative Neurology*, vol. 411, no. 4, pp. 578–590, 1999.

[44] J. Hellström, A. L. R. Oliveira, B. Meister, and S. Cullheim, "Large cholinergic nerve terminals on subsets of motoneurons and their relation to muscarinic receptor type 2," *The Journal of Comparative Neurology*, vol. 460, no. 4, pp. 476–486, 2003.

[45] J. I. Nagy, T. Yamamoto, and L. M. Jordan, "Evidence for the cholinergic nature of C-terminals associated with subsurface cisterns in alpha-motoneurons of rat," *Synapse*, vol. 15, no. 1, pp. 17–32, 1993.

[46] J. M. Wilson, J. Rempel, and R. M. Brownstone, "Postnatal development of cholinergic synapses on mouse spinal motoneurons," *The Journal of Comparative Neurology*, vol. 474, no. 1, pp. 13–23, 2004.

[47] G. B. Miles, R. Hartley, A. J. Todd, and R. M. Brownstone, "Spinal cholinergic interneurons regulate the excitability of motoneurons during locomotion," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 7, pp. 2448–2453, 2007.

[48] L. Zagoraiou, T. Akay, J. F. Martin, R. M. Brownstone, T. M. Jessell, and G. B. Miles, "A cluster of cholinergic premotor interneurons modulates mouse locomotor activity," *Neuron*, vol. 64, no. 5, pp. 645–662, 2009.

[49] D. B. Hoover, R. H. Baisden, and J. V. Lewis, "Axotomy-induced loss of m2 muscarinic receptor mRNA in the rat facial motor nucleus precedes a decrease in concentration of muscarinic receptors," *The Histochemical Journal*, vol. 28, no. 11, pp. 771–778, 1996.

[50] D. B. Hoover and J. C. Hancock, "Effect of facial nerve transection on acetylcholinesterase, choline acetyltransferase and [^{3}H] quinuclidinyl benzilate binding in rat facial nuclei," *Neuroscience*, vol. 15, no. 2, pp. 481–487, 1985.

[51] E. C. Witts, L. Zagoraiou, and G. B. Miles, "Anatomy and function of cholinergic C bouton inputs to motor neurons," *Journal of Anatomy*, vol. 224, no. 1, pp. 52–60, 2014.

[52] D. Gonzalez-Forero and B. Moreno-Lopez, "Retrograde response in axotomized motoneurons: nitric oxide as a key player in triggering reversion toward a dedifferentiated phenotype," *Neuroscience*, vol. 283, pp. 138–165, 2014.

[53] M. Kerzoncuf, L. Bensoussan, A. Delarque, J. Durand, J. M. Viton, and C. Rossi-Durand, "Plastic changes in spinal synaptic transmission following botulinum toxin A in patients with post-stroke spasticity," *Journal of Rehabilitation Medicine*, vol. 47, no. 10, pp. 910–916, 2015.

[54] R. Kumar, H. P. Dhaliwal, R. V. Kukreja, and B. R. Singh, "The botulinum toxin as a therapeutic agent: molecular structure and mechanism of action in motor and sensory systems," *Seminars in Neurology*, vol. 36, no. 01, pp. 010–019, 2016.

[55] R. Mazzocchio and M. Caleo, "More than at the neuromuscular synapse: actions of botulinum neurotoxin A in the central nervous system," *The Neuroscientist*, vol. 21, no. 1, pp. 44–61, 2015.

[56] G. Sandrini, R. De Icco, C. Tassorelli, N. Smania, and S. Tamburin, "Botulinum neurotoxin type A for the treatment of pain: not just in migraine and trigeminal neuralgia," *The Journal of Headache and Pain*, vol. 18, no. 1, p. 38, 2017.

[57] H. B. Rind, R. Butowt, and C. S. von Bartheld, "Synaptic targeting of retrogradely transported trophic factors in motoneurons: comparison of glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor, and cardiotrophin-1 with tetanus toxin," *The Journal of Neuroscience*, vol. 25, no. 3, pp. 539–549, 2005.

[58] J. Krakowiak, C. Liu, C. Papudesu, P. J. Ward, J. C. Wilhelm, and A. W. English, "Neuronal BDNF signaling is necessary

for the effects of treadmill exercise on synaptic stripping of axotomized motoneurons," *Neural Plasticity*, vol. 2015, Article ID 392591, 11 pages, 2015.

[59] M. Gao, P. Lu, D. Lynam et al., "BDNF gene delivery within and beyond templated agarose multi-channel guidance scaffolds enhances peripheral nerve regeneration," *Journal of Neural Engineering*, vol. 13, no. 6, article 066011, 2016.

[60] D. Santos, F. Gonzalez-Perez, X. Navarro, and J. Del Valle, "Dose-dependent differential effect of neurotrophic factors on in vitro and in vivo regeneration of motor and sensory neurons," *Neural Plasticity*, vol. 2016, Article ID 4969523, 13 pages, 2016.

[61] M. A. Davis-Lopez de Carrizosa, C. J. Morado-Diaz, S. Morcuende, R. R. de la Cruz, and A. M. Pastor, "Nerve growth factor regulates the firing patterns and synaptic composition of motoneurons," *The Journal of Neuroscience*, vol. 30, no. 24, pp. 8308–8319, 2010.

[62] P. M. Calvo, R. R. de la Cruz, and A. M. Pastor, "Synaptic loss and firing alterations in axotomized motoneurons are restored by vascular endothelial growth factor (VEGF) and VEGF-B," *Experimental Neurology*, vol. 304, pp. 67–81, 2018.

[63] K. W.-H. Lo, T. Jiang, K. A. Gagnon, C. Nelson, and C. T. Laurencin, "Small-molecule based musculoskeletal regenerative engineering," *Trends in Biotechnology*, vol. 32, no. 2, pp. 74–81, 2014.

[64] Y. Imai, I. Ibata, D. Ito, K. Ohsawa, and S. Kohsaka, "A novel gene iba1 in the major histocompatibility complex class III region encoding an EF hand protein expressed in a monocytic lineage," *Biochemical and Biophysical Research Communications*, vol. 224, no. 3, pp. 855–862, 1996.

[65] D. Ito, Y. Imai, K. Ohsawa, K. Nakajima, Y. Fukuuchi, and S. Kohsaka, "Microglia-specific localisation of a novel calcium binding protein, Iba1," *Molecular Brain Research*, vol. 57, no. 1, pp. 1–9, 1998.

[66] F. A. Chaudhry, R. J. Reimer, E. E. Bellocchio et al., "The vesicular GABA transporter, VGAT, localizes to synaptic vesicles in sets of glycinergic as well as GABAergic neurons," *The Journal of Neuroscience*, vol. 18, no. 23, pp. 9733–9750, 1998.

[67] S. L. McIntire, R. J. Reimer, K. Schuske, R. H. Edwards, and E. M. Jorgensen, "Identification and characterization of the vesicular GABA transporter," *Nature*, vol. 389, no. 6653, pp. 870–876, 1997.

[68] S. M. Wojcik, S. Katsurabayashi, I. Guillemin et al., "A shared vesicular carrier allows synaptic corelease of GABA and glycine," *Neuron*, vol. 50, no. 4, pp. 575–587, 2006.

[69] F. J. Alvarez, R. M. Villalba, R. Zerda, and S. P. Schneider, "Vesicular glutamate transporters in the spinal cord, with special reference to sensory primary afferent synapses," *The Journal of Comparative Neurology*, vol. 472, no. 3, pp. 257–280, 2004.

[70] A. L. R. Oliveira, F. Hydling, E. Olsson et al., "Cellular localization of three vesicular glutamate transporter mRNAs and proteins in rat spinal cord and dorsal root ganglia," *Synapse*, vol. 50, no. 2, pp. 117–129, 2003.

6. Discussion

The results of this study strongly support the hypothesis that the degree of preservation/recovery of synaptic inputs to regenerated motoneurons, in particular cholinergic perisomatic terminals, influences functional recovery after peripheral nerve injury. In addition, the work demonstrates, for the first time, that drug application to the acutely severed peripheral nerve is a feasible approach to manipulate post-lesion synaptic plasticity and functional recovery.

6.1 Methodological considerations

The femoral nerve model in mice and rats has been widely used for regeneration studies (Irintchev, 2011). It has been initially employed as a model system to study specificity of motor reinnervation in a choice situation (mixed quadriceps versus purely sensory saphenous nerve branch) using retrograde tracing methods (Brushart, 1988, 1993). Substantial enrichment of the model was the design of an objective numerical test for assessment of quadriceps motor function, the single frame motion analysis (SFMA, Irintchev et al., 2005b). The combination of SFMA and morphological methods like retrograde tracing, axonal morphometry and synaptic terminal counts gives the opportunity to search for variables determining the outcome of nerve regeneration (Eberhardt et al., 2006; Ahlborn et al., 2007; Mehanna et al., 2010; Guseva et al., 2009, 2011, 2018) . The SFMA method is validated for rats and characterized by high precision, reliability and reproducibility (Kruspe et al., 2014). The SFMA results in this experiment are in full agreement with previous data on the course and outcome of femoral nerve section/suture (Kruspe et al., 2014). The analyses were performed in a blind fashion and the coefficients of variation (standard deviation / mean group value, a measure of precision and repeatability of an assay) for individual data sets (animal group on particular postoperative day) were typically as low as 2% - 7%. Therefore, it can be assumed that the results on functional recovery in this study are reliable.

Another technique of major importance for this experimental study was the stereological assessment of cell and synaptic terminal densities. This approach, in particular the optical disector method, also has a long tradition in our laboratory and has been successfully applied in studies on different CNS areas of rodents (Irintchev et al., 2005a; Nikonenko et al., 2006; Jakovcevski et al., 2009; Tereshchenko et al., 2011; Hundeshagen et al., 2013; Raslan et al., 2014). The optical disector enables

precise (“unbiased”) estimates of object densities based on systematic random sampling of sections with a three-dimensional probe (Howard and Reed, 1998). Using standardized immunohistochemical protocols on spaced serial sections and optical disector, densities of different structures, e.g. different types of cells or synaptic terminals, can be estimated in the same reference volume and are, thus, comparable to each other (Irintchev et al., 2005a; Raslan et al., 2014). Blinded analyses, rigorous application of stereological principles and high-quality immunofluorescence stainings were the prerequisites for generating reliable numerical data in our present experiments. At the same time it has to be underlined that we estimated densities of synaptic terminals, i.e. presynaptic elements, and this is not automatically equivalent to densities of synapses consisting of pre- and postsynaptic elements. This means that, for example, the number of terminals “detached”, i.e. separated by thin astrocytic lamellae, from their post-synaptic counterparts during post-traumatic synaptic elimination, cannot be identified and counted separately from “attached” terminals, i.e. synapse-forming ones. Electron microscopy alone or in combination with immunohistochemistry provides the opportunity to identify synapses of defined type. However, this procedure is extremely labor intensive and the sample sizes that can be achieved, often limited to a few synapses in an animal group, might not be representative (Brannstrom and Kellerth, 1998; Brännström and Kellerth, 1999). Therefore, analyses of synaptic terminals and not synapses are currently standard procedures in many laboratories (Davis-Lopez de Carrizosa et al., 2010; Berg et al., 2012; Mehanna et al., 2014; Chawla et al., 2017; Schultz et al., 2017).

6.2 Drug effects on synaptic terminal numbers

Prior to the experiments it was hypothesized that BDNF would have synaptotrophic effects and, indeed, the results showed that injury-related loss of $ChAT^+$ perisomatic boutons is prevented at 1 week. At 2 months after injury, however, the effect was no longer present. Also excitatory $VGLUT2^+$ and inhibitory $VGAT^+$ terminals were affected at neither 1 week nor 2 months, and $VGLUT1^+$ terminals were even reduced at 2 months after injury (Table 2 in 5.). It is possible that the intracellular concentration of active exogenous BDNF achieved in our experiment has not been optimal to produce pronounced, long-term effects. BDNF appears to have a dose-dependent influence on nerve regeneration, i.e. facilitation at low doses and inhibition

at higher ones (Richner et al., 2014). Therefore, future dose-dependence studies are required to properly assess the value of BDNF as a regeneration promoting agent in our model.

In contrast to BDNF, BoNT was expected to enhance loss of terminals after axotomy with a more pronounced effect on excitatory ($VGLUT1^+$ and $VGLUT2^+$) than on inhibitory ($VGAT^+$) terminals (Verderio et al., 2004, 2007). Such effects were not found at 2 months after injury. Surprisingly, the only significant, long-lasting effect, on $ChAT^+$ terminals, was synaptotrophic (Table 2 in 5.). Such an effect cannot be explained by the known major mechanism of BoNT action – inhibition of synaptic transmission via cleaving of SNAP-25 (Verderio et al., 2004). A possible mechanism underlying this effect is partial inhibition and reactive sprouting of the cholinergic input to motoneurons. BoNT neurotrophic effects could possibly be also achieved via co-localization and signaling through the p75 receptor (Restani et al., 2012a; Cobianchi et al., 2017). Only future experiments can reveal the true mechanism(s) underlying the unexpected synaptotrophic action of BoNT.

6.3 Mechanisms underlying enhanced functional recovery

We applied BoNT only once using the time frame between axonal membrane damage and sealing to load the proximal axon and cell body with toxin similar to retrograde tracers. Our expectation was that this uptake will be sufficient to "prime" the initial responses of motoneurons to injury, in particular their deafferentation, and, thus, eventually achieve long-term effects on regeneration without need of repeated drug delivery to the injury site. As estimated by gait analysis, our experiment was successful since functional regeneration was enhanced already at two weeks after injury and recovery remained accelerated for months thereafter. Enhancement of axonal regrowth in the crushed sciatic nerve of mice by a single low-dose intraneural application of BoNT has been just reported but the underlying mechanism(s) for these effects have remained unclear (Cobianchi et al., 2017). Here we propose that the improvement of regeneration in our model is a consequence of attenuated loss of cholinergic modulatory input to femoral motoneurons (see 6.4). In addition, it is possible that BoNT has an additional neuroprotective effect. At one week after injury we found, compared with control rats, an increase in $VGAT^+$ inhibitory afferents in the quadriceps motor nucleus, reduced numbers of excitatory $VGLUT1^+$ Ia afferents and no change in excitatory $VGLUT2^+$ terminals (Table 2 in 5.). We can speculate that

this constellation attenuates the increased excitability of the axotomized motoneurons and, thus, allows better recovery of the motoneuron and its better regeneration (Davis-Lopez de Carrizosa et al., 2009; Gonzalez-Forero and Moreno-Lopez, 2014). It is also thinkable that BoNT-related modulations of reflexes and/or pain-related transmission may have also positive functional consequences (Kerzoncuf et al., 2015; Mazzocchio and Caleo, 2015; Kumar et al., 2016; Sandrini et al., 2017).

6.4 Perisomatic C-type cholinergic synapses

This work linked better functional recovery with higher numbers of cholinergic perisomatic terminals in BoNT-treated animals. The correlations between $ChAT^+$ terminal numbers and functional parameters were strong ($r^2 \sim 0.70$), in contrast to other variables studied, other types of synapses and preferential motor reinnervation. In addition, previous work using facial nerve or spinal cord injury models has also shown strong statistical relationships between degree of functional recovery, on one side, and degree of preservation/recovery of $ChAT^+$ terminal frequency on facial (Hundeshagen et al., 2013; Raslan et al., 2014) or spinal motoneurons (Apostolova et al., 2006; Jakovcevski et al., 2007; Lee et al., 2009), on the other side. These large cholinergic terminals form C-type synapses on motoneuronal perikarya and proximal dendrites and utilize M2 muscarinic receptors for acetylcholine in the postsynaptic membrane (Davidoff and Irintchev, 1986; Nagy et al., 1993; Hellstrom et al., 1999; Hellström et al., 2003; Wilson et al., 2004; Csaba et al., 2013). Although not that numerous, these synapses strongly influence motoneuron function by regulating action potential afterhyperpolarization in a way that, under normal conditions, ensures sufficient motoneuron output to drive motor behavior (Miles et al., 2007; Zagoraiou et al., 2009). It can be, therefore, assumed that partial loss of perisomatic cholinergic terminals, associated with a reduced expression of postsynaptic receptors (Hoover and Hancock, 1985; Hoover et al., 1996), may significantly impair motor behaviors such as walking, whisking and blinking (Witts et al., 2014).

7. Conclusions and perspectives

The results of this study suggest that preservation of the C-terminal input to motoneurons favors functional recovery after nerve injury and regeneration. It is encouraging that a single intraoperative drug application can lead to long lasting positive functional effects. Many different agents could also be useful tools to modify neuronal responses to axotomy and improve functional outcome: neurotrophin-3 (Davis-Lopez de Carrizosa et al., 2009), NGF (Davis-Lopez de Carrizosa et al., 2010), vascular endothelial growth factor, VEGF (Calvo et al., 2018) or small bioactive molecules (Lo et al., 2014). Although BoNT is widely used in contemporary medicine, further experimental research is required before it can be considered as a potential “regeneration drug” for humans suffering peripheral nerve injuries.

8. References

Ahlborn P, Schachner M, Irintchev A. 2007. One hour electrical stimulation accelerates functional recovery after femoral nerve repair. Exp Neurol 208:137-144.

Al-Majed A, Brushart T, Gordon T. 2000a. Electrical stimulation accelerates and increases expression of BDNF and trkB mRNA in regenerating rat femoral motoneurons. Eur J Neurosci 12:4381-4390.

Al-Majed A, Neumann C, Brushart T, Gordon T. 2000b. Brief electrical stimulation promotes the speed and accuracy of motor axonal regeneration. J Neurosci 20:2602-2608.

Antonucci F, Rossi C, Gianfranceschi L, Rossetto O, Caleo M. 2008. Long-distance retrograde effects of botulinum neurotoxin A. J Neurosci 28:3689-3696.

Apostolova I, Irintchev A, Schachner M. 2006. Tenascin-R restricts posttraumatic remodeling of motoneuron innervation and functional recovery after spinal cord injury in adult mice. J Neurosci 26:7849-7859.

Berg A, Zelano J, Stephan A, Thams S, Barres BA, Pekny M, Pekna M, Cullheim S. 2012. Reduced removal of synaptic terminals from axotomized spinal motoneurons in the absence of complement C3. Exp Neurol 237:8-17.

Blinzinger K, Kreutzberg G. 1968. Displacement of synaptic terminals from regenerating motoneurons by microglial cells. Z Zellforsch Mikrosk Anat 85:145-157.

Borke RC, Bridwell RS, Nau ME. 1995. The progression of deafferentation as a retrograde reaction to hypoglossal nerve injury. J Neurocytol 24:763-774.

Brännström T, Kellerth JO. 1998. Changes in synaptology of adult cat spinal alpha-motoneurons after axotomy. Exp Brain Res 118:1-13.

Brännström T, Kellerth JO. 1999. Recovery of synapses in axotomized adult cat spinal motoneurons after reinnervation into muscle. Exp Brain Res 125:19-27.

Brushart T. 1988. Preferential reinnervation of motor nerves by regenerating motor axons. J Neurosci 8:1026-1031.

Brushart T. 1993. Motor axons preferentially reinnervate motor pathways. J Neurosci 13:2730-2738.

Calvo PM, de la Cruz RR, Pastor AM. 2018. Synaptic loss and firing alterations in Axotomized Motoneurons are restored by vascular endothelial growth factor (VEGF) and VEGF-B. Exp Neurol 304:67-81.

Chawla RS, Zuchner M, Mastrangelopoulou M, Lambert FM, Glover JC, Boulland JL. 2017. Cellular reactions and compensatory tissue re-organization during spontaneous recovery after spinal cord injury in neonatal mice. Dev Neurobiol 77:928-946.

Chen DH. 1978. Qualitative and quantitative study of synaptic displacement in chromatolyzed spinal motoneurons of the cat. J Comp Neurol 177:635-664.

Cobianchi S, Jaramillo J, Luvisetto S, Pavone F, Navarro X. 2017. Botulinum neurotoxin A promotes functional recovery after peripheral nerve injury by increasing regeneration of myelinated fibers. Neuroscience 359:82-91.

Csaba Z, Krejci E, Bernard V. 2013. Postsynaptic muscarinic m2 receptors at cholinergic and glutamatergic synapses of mouse brainstem motoneurons. J Comp Neurol 521:2008-2024.

Davidoff M, Irintchev A. 1986. Acetylcholinesterase activity and type C synapses in the hypoglossal, facial and spinal-cord motor nuclei of rats. An electron-microscope study. Histochemistry 84:515-524.

Davis-Lopez de Carrizosa MA, Morado-Diaz CJ, Morcuende S, de la Cruz RR, Pastor AM. 2010. Nerve growth factor regulates the firing patterns and synaptic composition of motoneurons. J Neurosci 30:8308-8319.

Davis-Lopez de Carrizosa MA, Morado-Diaz CJ, Tena JJ, Benitez-Temino B, Pecero ML, Morcuende SR, de la Cruz RR, Pastor AM. 2009. Complementary actions of BDNF and neurotrophin-3 on the firing patterns and synaptic composition of motoneurons. J Neurosci 29:575-587.

Eberhardt K, Irintchev A, Al-Majed A, Simova O, Brushart T, Gordon T, Schachner M. 2006. BDNF/TrkB signaling regulates HNK-1 carbohydrate expression in regenerating motor nerves and promotes functional recovery after peripheral nerve repair. Exp Neurol 198:500-510.

Gonzalez-Forero D, Moreno-Lopez B. 2014. Retrograde response in axotomized motoneurons: nitric oxide as a key player in triggering reversion toward a dedifferentiated phenotype. Neuroscience 283:138-165.

Graeber MB, Bise K, Mehraein P. 1993. Synaptic stripping in the human facial nucleus. Acta Neuropathol 86:179-181.

Guseva D, Angelov DN, Irintchev A, Schachner M. 2009. Ablation of adhesion molecule L1 in mice favours Schwann cell proliferation and functional recovery after peripheral nerve injury. Brain 132:2180-2195.

Guseva D, Jakovcevski I, Irintchev A, Leshchyns'ka I, Sytnyk V, Ponimaskin E, Schachner M. 2018. Cell adhesion molecule close homolog of L1 (CHL1) guides the regrowth of regenerating motor axons and regulates synaptic coverage of motor neurons. Front Mol Neurosci 11:174.

Guseva D, Zerwas M, Xiao MF, Jakovcevski I, Irintchev A, Schachner M. 2011. Adhesion molecule L1 overexpressed under the control of the neuronal Thy-1 promoter improves myelination after peripheral nerve injury in adult mice. Exp Neurol 229:339-352.

Heckman CJ, Mottram C, Quinlan K, Theiss R, Schuster J. 2009. Motoneuron excitability: the importance of neuromodulatory inputs. Clin Neurophysiol 120:2040-2054.

Hellström J, Arvidsson U, Elde R, Cullheim S, Meister B. 1999. Differential expression of nerve terminal protein isoforms in VAChT-containing varicosities of the spinal cord ventral horn. J Comp Neurol 411:578-590.

Hellström J, Oliveira AL, Meister B, Cullheim S. 2003. Large cholinergic nerve terminals on subsets of motoneurons and their relation to muscarinic receptor type 2. J Comp Neurol 460:476-486.

Hoover DB, Baisden RH, Lewis JV. 1996. Axotomy-induced loss of m2 muscarinic receptor mRNA in the rat facial motor nucleus precedes a decrease in concentration of muscarinic receptors. Histochem J 28:771-778.

Hoover DB, Hancock JC. 1985. Effect of facial nerve transection on acetylcholinesterase, choline acetyltransferase and [3H]quinuclidinyl benzilate binding in rat facial nuclei. Neuroscience 15:481-487.

Howard V, Reed MG. 1998. Unbiased stereology : three-dimensional measurement in microscopy. New York: Springer.

Hundeshagen G, Szameit K, Thieme H, Finkensieper M, Angelov DN, Guntinas-Lichius O, Irintchev A. 2013. Deficient functional recovery after facial nerve crush in rats is associated with restricted rearrangements of synaptic terminals in the facial nucleus. Neuroscience 248C:307-318.

Irintchev A. 2011. Potentials and limitations of peripheral nerve injury models in rodents with particular reference to the femoral nerve. Ann Anat 193:276-285.

Irintchev A, Rollenhagen A, Troncoso E, Kiss JZ, Schachner M. 2005a. Structural and functional aberrations in the cerebral cortex of tenascin-C deficient mice. Cereb Cortex 15:950-962.

Irintchev A, Simova O, Eberhardt KA, Morellini F, Schachner M. 2005b. Impacts of lesion severity and tyrosine kinase receptor B deficiency on functional outcome of femoral nerve injury assessed by a novel single-frame motion analysis in mice. Eur J Neurosci 22:802-808.

Jakovcevski I, Siering J, Hargus G, Karl N, Hoelters L, Djogo N, Yin S, Zecevic N, Schachner M, Irintchev A. 2009. Close homologue of adhesion molecule L1 promotes survival of Purkinje and granule cells and granule cell migration during murine cerebellar development. J Comp Neurol 513:496-510.

Jakovcevski I, Wu J, Karl N, Leshchyns'ka I, Sytnyk V, Chen J, Irintchev A, Schachner M. 2007. Glial scar expression of CHL1, the close homolog of the adhesion molecule L1, limits recovery after spinal cord injury. J Neurosci 27:7222-7233.

Kerns JM, Hinsman EJ. 1973a. Neuroglial response to sciatic neurectomy. I. Light microscopy and autoradiography. J Comp Neurol 151:237-254.

Kerns JM, Hinsman EJ. 1973b. Neuroglial response to sciatic neurectomy. II. Electron microscopy. J Comp Neurol 151:255-280.

Kerzoncuf M, Bensoussan L, Delarque A, Durand J, Viton JM, Rossi-Durand C. 2015. Plastic changes in spinal synaptic transmission following botulinum toxin A in patients with post-stroke spasticity. J Rehabil Med 47:910-916.

Kreutzberg GW. 1993. Dynamic changes in motoneurons during regeneration. Restor Neurol Neurosci 5:59-60.

Kruspe M, Thieme H, Guntinas-Lichius O, Irintchev A. 2014. Motoneuron regeneration accuracy and recovery of gait after femoral nerve injuries in rats. Neuroscience 280:73-87.

Kumar R, Dhaliwal HP, Kukreja RV, Singh BR. 2016. The botulinum toxin as a therapeutic agent: Molecular structure and mechanism of action in motor and sensory systems. Semin Neurol 36:10-19.

Lee HJ, Jakovcevski I, Radonjic N, Hoelters L, Schachner M, Irintchev A. 2009. Better functional outcome of compression spinal cord injury in mice is associated with enhanced H-reflex responses. Exp Neurol 216:365-374.

Lo KW, Jiang T, Gagnon KA, Nelson C, Laurencin CT. 2014. Small-molecule based musculoskeletal regenerative engineering. Trends Biotech 32:74-81.

Matak I, Riederer P, Lackovic Z. 2012. Botulinum toxin's axonal transport from periphery to the spinal cord. Neurochem Int 61:236-239.

Mazzocchio R, Caleo M. 2015. More than at the neuromuscular synapse: actions of botulinum neurotoxin A in the central nervous system. Neuroscientist 21:44-61.

Mehanna A, Jakovcevski I, Acar A, Xiao MF, Loers G, Rougon G, Irintchev A, Schachner M. 2010. Polysialic acid glycomimetic promotes functional recovery and plasticity after spinal cord injury in mice. Mol Ther 18:34-43.

Mehanna A, Szpotowicz E, Schachner M, Jakovcevski I. 2014. Improved regeneration after femoral nerve injury in mice lacking functional T- and B-lymphocytes. Exp Neurol 261:147-155.

Miles GB, Hartley R, Todd AJ, Brownstone RM. 2007. Spinal cholinergic interneurons regulate the excitability of motoneurons during locomotion. Proc Natl Acad Sci U S A 104:2448-2453.

Nagy JI, Yamamoto T, Jordan LM. 1993. Evidence for the cholinergic nature of C-terminals associated with subsurface cisterns in alpha-motoneurons of rat. Synapse 15:17-32.

Nikonenko A, Sun M, Lepsveridze E, Apostolova I, Petrova I, Irintchev A, Dityatev A, Schachner M. 2006. Enhanced perisomatic inhibition and impaired long-term potentiation in the CA1 region of juvenile CHL1-deficient mice. Eur J Neurosci 23:1839-1852.

Novikov LN, Novikova LN, Holmberg P, Kellerth J. 2000. Exogenous brain-derived neurotrophic factor regulates the synaptic composition of axonally lesioned and normal adult rat motoneurons. Neuroscience 100:171-181.

Pastor AM, Moreno-Lopez B, De La Cruz RR, Delgado-Garcia JM. 1997. Effects of botulinum neurotoxin type A on abducens motoneurons in the cat: ultrastructural and synaptic alterations. Neuroscience 81:457-478.

Raslan A, Ernst P, Werle M, Thieme H, Szameit K, Finkensieper M, Guntinas-Lichius O, Irintchev A. 2014. Reduced cholinergic and glutamatergic synaptic input to regenerated motoneurons after facial nerve repair in rats: Potential implications for recovery of motor function. Brain Struct Funct 219:891-909.

Restani L, Giribaldi F, Manich M, Bercsenyi K, Menendez G, Rossetto O, Caleo M, Schiavo G. 2012a. Botulinum neurotoxins A and E undergo retrograde axonal transport in primary motor neurons. PLoS Pathogens 8:e1003087.

Restani L, Novelli E, Bottari D, Leone P, Barone I, Galli-Resta L, Strettoi E, Caleo M. 2012b. Botulinum neurotoxin A impairs neurotransmission following retrograde transynaptic transport. Traffic 13:1083-1089.

Richner M, Ulrichsen M, Elmegaard SL, Dieu R, Pallesen LT, Vaegter CB. 2014. Peripheral nerve injury modulates neurotrophin signaling in the peripheral and central nervous system. Mol Neurobiol 50:945-970.

Rotterman TM, Nardelli P, Cope TC, Alvarez FJ. 2014. Normal distribution of VGLUT1 synapses on spinal motoneuron dendrites and their reorganization after nerve injury. J Neurosci 34:3475-3492.

Sandrini G, De Icco R, Tassorelli C, Smania N, Tamburin S. 2017. Botulinum neurotoxin type A for the treatment of pain: not just in migraine and trigeminal neuralgia. J Headache Pain 18:38.

Schultz AJ, Rotterman TM, Dwarakanath A, Alvarez FJ. 2017. VGLUT1 synapses and P-boutons on regenerating motoneurons after nerve crush. J Comp Neurol 525:2876-2889.

Spejo AB, Oliveira AL. 2015. Synaptic rearrangement following axonal injury: Old and new players. Neuropharmacology 96:113-123.

Sumner BE. 1975. A quantitative analysis of boutons with different types of synapse in normal and injured hypoglossal nuclei. Exp Neurol 49:406-417.

Sumner BE, Sutherland FI. 1973. Quantitative electron microscopy on the injured hypoglossal nucleus in the rat. J Neurocytol 2:315-328.

Tereshchenko Y, Morellini F, Dityatev A, Schachner M, Irintchev A. 2011. Neural Cell Adhesion Molecule Ablation in Mice Causes Hippocampal Dysplasia and Loss of Septal Cholinergic Neurons. J Comp Neurol 519:2475-2492.

Verderio C, Grumelli C, Raiteri L, Coco S, Paluzzi S, Caccin P, Rossetto O, Bonanno G, Montecucco C, Matteoli M. 2007. Traffic of botulinum toxins A and E in excitatory and inhibitory neurons. Traffic 8:142-153.

Verderio C, Pozzi D, Pravettoni E, Inverardi F, Schenk U, Coco S, Proux-Gillardeaux V, Galli T, Rossetto O, Frassoni C, Matteoli M. 2004. SNAP-25 modulation of calcium dynamics underlies differences in GABAergic and glutamatergic responsiveness to depolarization. Neuron 41:599-610.

Wilson JM, Rempel J, Brownstone RM. 2004. Postnatal development of cholinergic synapses on mouse spinal motoneurons. J Comp Neurol 474:13-23.

Witts EC, Zagoraiou L, Miles GB. 2014. Anatomy and function of cholinergic C bouton inputs to motor neurons. J Anat 224:52-60.

Zagoraiou L, Akay T, Martin JF, Brownstone RM, Jessell TM, Miles GB. 2009. A cluster of cholinergic premotor interneurons modulates mouse locomotor activity. Neuron 64:645-662.

9. Anhänge (Appendices)

9.1 Lebenslauf (curriculum vitae)

9.2 Ehrenwörtliche Erklärung (Statement)

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: PD Dr. A. Irintchev, Prof. O. Guntinas-Lichius

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena,10.06.2019 Unterschrift des Verfassers

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